

Biological Test Method

**Sublethal Toxicity Tests
to Assess Sediments
Intended for Disposal at Sea**

May 2001

Marine Environment Division
Toxics Pollution Prevention Directorate
Environment Canada

National Library of Canada cataloguing in publication data

Main entry under title :

Biological test method. Sublethal toxicity tests to assess sediments intended for disposal at sea

(Report ; EPS 1/RM/40)

Issued also in French under title : Méthode d'essai biologique.

Essais de toxicité sublétales en vue de l'évaluation des sédiments destinés à l'immersion en mer.

Includes bibliographical references.

ISBN 0-662-30689-9

Cat. No. En49-24/1-40E

1. Environmental toxicology -- Environmental aspects.
 2. Aquatic biology -- Environmental aspects.
 3. Waste disposal in the ocean -- Environmental aspects.
- I. Canada. Environment Canada.
 - II. Canada. Marine Environment Division.

QH90.8B40 2001

363.739'4



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Readers' Comments

Comments regarding the content of this document should be addressed to:

Head, Disposal at Sea Program
Marine Environment Division
Toxics Pollution Prevention Directorate
Environmental Protection Service
Environment Canada
Ottawa, Ontario
K1A 0H3

tel.: (819) 953-2264

fax: (819) 953-0913

Cette publication est aussi disponible en français sous le titre : *Méthode d'essai biologique : Essais de toxicité sublétale en vue de l'évaluation des sédiments destinés à l'immersion en mer*. Pour l'obtenir, s'adresser à :

Publications de la Protection de l'environnement
Service de la Protection de l'environnement
Environnement Canada
Ottawa (Ontario)
K1A 0H3

courriel : epspubs@ec.gc.ca



Abstract

This report describes methods recommended by Environment Canada for performing sublethal tests for the assessment of marine or estuarine sediments intended for disposal at sea. The tests assembled here are selected from peer-reviewed methods which may also be published independently for other purposes. This document provides a convenient consolidation of suitable sublethal methods and allows for a better vehicle to tailor methods specifically for ocean disposal assessment.

Each chapter in this report provides a complete method that can be used independently from the next. Within each chapter is a description of the test and of the species that can be used. Procedures are given for holding test species in the laboratory and preparing them for the testing phase. Conditions and procedures are outlined for testing sediments. Instructions are included for test facilities, handling and storing samples, preparing test solutions and initiating tests, specific test conditions, appropriate observations and measurements, endpoints and methods of calculations and validation of the test.

Résumé

Le présent document expose les méthodes d'essai sublétal recommandées par Environnement Canada en vue de l'évaluation des sédiments marins ou estuariens destinés à l'immersion en mer. Les essais présentés ci-dessus ont été choisis parmi des méthodes qui ont été soumises à un examen par les pairs; ils peuvent aussi faire l'objet d'une publication sous titre séparé à d'autres fins. Ce document rassemble des méthodes d'essai sublétal convenant à l'évaluation des sédiments et permet d'adapter les méthodes en vue de l'immersion en mer.

Chaque chapitre de ce rapport présente une méthode complète qui peut être utilisée indépendamment de l'autre. Dans chaque chapitre, on décrit l'essai et les espèces recommandées. Le document présente également les modes de garde des espèces en laboratoire et de préparation de ces espèces en vue des essais. Les conditions et procédures d'essais sur les sédiments sont décrites. Le lecteur trouvera des directives concernant les installations d'essai, la manipulation et le stockage des échantillons, la préparation des solutions d'essai, la mise en route des essais, les conditions particulières, les observations et mesures appropriées, les mesures terminales, les méthodes de calcul et la validation de l'essai.

Foreword

Biological Test Method: Sublethal toxicity tests to assess sediments intended for disposal at sea. (EPS 1/RM/40).

This method is a consolidated document in two parts that present two current sublethal toxicity test methods in their entirety which are suitable for use to assess sediments intended for disposal at sea. These methods will be amended from time to time. Any amendments will be on the CEPA Registry at www.ec.gc.ca/CEPARRegistry.

They are:

Part 1. Biological Test Method: Toxicity Test Using Luminescent Bacteria (*Photobacterium phosphoreum*). Also published separately as: EPS 1/RM/24.

Part 2. Biological Test Method: Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars). Also published separately as: EPS 1/RM/27.

These reports are intended to provide guidance and to facilitate the use of consistent, appropriate and comprehensive procedures for obtaining data on toxicity to aquatic life of specific test substances.



Environmental Protection Series



Biological Test Method:
Toxicity Test Using
Luminescent Bacteria
(*Photobacterium
phosphoreum*)

Report EPS 1/RM/24
November 1992

Canada



Environment
Canada

Environnement
Canada

**Biological Test Method:
Toxicity Test
Using Luminescent Bacteria
(*Photobacterium phosphoreum*)**

Environmental Protection
Conservation and Protection
Environment Canada

Report EPS 1/RM/24
November 1992

CANADIAN CATALOGUING IN PUBLICATION DATA

Main entry under title:

Biological test method. Toxicity test using
luminescent bacteria (*Photobacterium phosphoreum*)

(Report ; EPS 1/RM/24)

Issued also in French under title: Méthode d'essai
biologique. Essai de toxicité sur la bactérie
luminescente (*Photobacterium phosphoreum*).

Includes bibliographical references.

ISBN 0-662-20379-8

DSS cat. no. En49-24/24E

1. *Photobacterium phosphoreum* -- Testing.
2. Aquatic organisms -- Effect of water pollution
on -- Testing -- Methodology -- Standards -- Canada.
3. Effluent quality -- Testing -- Methodology --
Standards -- Canada. 4. Toxicity testing --
Methodology -- Standards -- Canada. I. Canada.
Environmental Protection Directorate. II. Canada.
Environment Canada. III. Title: Toxicity test
using luminescent bacteria (*Photobacterium phosphoreum*).
- IV. Series: Report (Canada. Environment Canada) ;
EPS 1/RM/24.

QR82.V53B56 1993

589.9'5

C93-099465-5

Readers' Comments

Comments regarding the content of this report should be addressed to:

Richard Scroggins
Technology Development Branch
Environment Canada
Ottawa, Ontario
K1A 0H3

Gary Sergy
Technology Development Branch
Environment Canada
No. 210, 4999-98 Avenue
Edmonton, Alberta
T6B 2X3

Cette publication est aussi disponible en français. Pour l'obtenir, s'adresser à:

Publications de la Protection de l'environnement
Conservation et Protection
Environnement Canada
Ottawa (Ontario)
K1A 0H3



Abstract

Methods recommended by Environment Canada for performing toxicity tests with the luminescent bacterium Photobacterium phosphoreum, are described in this report.

General or universal conditions and procedures are outlined for testing a variety of substances. Additional conditions and procedures are stipulated that are specific for assessing samples of chemical, effluent, leachate, elutriate, receiving water, and sediment or other solids such as soil. Included are instructions on sample handling and storage, test facility requirements, procedures for preparing test solutions and initiating tests, specified test conditions, appropriate observations and measurements, endpoints, methods of calculation, and the use of reference toxicants.

The endpoint of the test is the concentration of sample which is estimated to cause 50% inhibition of light production by the bacteria (i.e., the IC50). This could be estimated after exposures of 5, 15, or 30 minutes.

Résumé

Le présent document expose les méthodes recommandées par Environnement Canada pour l'exécution d'essais de toxicité sur la bactérie luminescente Photobacterium phosphoreum.

Il présente les conditions et méthodes générales ou universelles permettant de réaliser des essais sur un large éventail de substances. On y précise aussi d'autres conditions et méthodes propres à l'évaluation d'échantillons de produits chimiques, d'effluents, de lixiviats, d'élutriats, de milieux récepteurs et de sédiments ou d'autres solides tels que des sols. Le lecteur y trouvera des instructions concernant la manipulation et le stockage des échantillons, les installations d'essai, la préparation des solutions d'essai et la mise en route des essais, les conditions prescrites pour les essais, les observations et mesures appropriées, les résultats des essais, les méthodes de calcul et l'utilisation de produits toxiques de référence.

Le résultat de l'essai est la concentration de l'échantillon qu'on estime qui cause une inhibition de 50 % de la production de lumière par la bactérie (c'est-à-dire la CI_{50}). L'estimation de cette valeur peut se faire après 5, 15 ou 30 minutes d'exposition.

Foreword

*This is one of a series of **recommended methods** for measuring and assessing the aquatic biological effects of toxic substances. Recommended methods are those which have been evaluated by Conservation and Protection (C&P), and are favoured:*

- for use in C&P aquatic toxicity laboratories;*
- for testing which is contracted out by Environment Canada or requested from outside agencies or industry;*
- in the absence of more specific instructions, such as are contained in regulations; and*
- as a foundation for the provision of very explicit instructions as might be required in a regulatory program or standard reference method.*

The different types of tests included in this series were selected on the basis of their acceptability for the needs of programs for environmental protection and management carried out by Environment Canada. These documents are intended to provide guidance and to facilitate the use of consistent, appropriate, and comprehensive procedures for obtaining data on toxic effects of samples of chemical, effluent, leachate, elutriate, receiving water, and sediment or similar solid.

Mention of trade names in this document does not constitute endorsement by Environment Canada; other products with similar value are available.



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List of Abbreviations and Chemical Formulae

ATP	adenosine triphosphate
°C	degree(s) Celsius
cm	centimetre(s)
d	day(s)
DO	dissolved oxygen (concentration)
EC ₅₀	median effective concentration
g	gram(s)
g/kg	grams per kilogram
h	hour(s)
HCl	hydrochloric acid
H ₂ O	water
IC _p	inhibiting concentration for a (specified) percent effect
IC ₅₀	50% inhibiting concentration
L	litre(s)
LC ₅₀	median lethal concentration
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
mm	millimetre(s)
MOAS	Microtox Osmotic Adjustment Solution
mS	millisiemen(s)
N	Normal
NaCl	sodium chloride

NaOH		sodium hydroxide
Recon		Reconstitution Solution
SD		standard deviation
sp.		species
TIE		toxicity identification evaluation
TM (™)		trade mark
µg		microgram
µL		microlitre
>		greater than
<		less than
≥		greater than or equal to
≤		less than or equal to
±		plus or minus
%		percentage or percent

Terminology

Note: all definitions have been given in the context of the procedures in this report, and might not be appropriate in another context.

Grammatical Terms

Must is used to express an absolute requirement.

Should is used to state that the specified condition or procedure is recommended and ought to be met if possible.

May is used to mean "is (are) allowed to".

Can is used to mean "is (are) able to".

Might is used to express the possibility that something could exist or happen.

General Technical Terms

Bioluminescence is a phenomenon of light emitted from living organisms as a result of their biochemical activities, usually enzymatic.

Compliance means in accordance with governmental permitting or regulatory requirements.

Conductivity is a numerical expression of the ability of an aqueous solution to carry an electric current. This ability depends on the concentrations of ions in solution, their valence and mobility, and on the solution's temperature. Conductivity in fresh waters is normally reported in the SI unit of millisiemens/metre, or as micromhos/centimetre ($1 \text{ mS/m} = 10 \text{ } \mu\text{mhos/cm}$). Conductivity is a standard method for measuring salinity (*q.v.*), with a result which is usually read off as g/kg or "parts per thousand".

Dispersant means a substance which reduces the surface tension between water and a hydrophobic substance (e.g., oil), thereby facilitating the dispersal of the hydrophobic material throughout the water as an emulsion.

Emulsifier means a substance that aids the fine mixing (in the form of small droplets) within water, of an otherwise hydrophobic substance.

Flocculation is the formation of a light, loose agglomeration of particles (i.e., a floc) from a solution.

Hardness is the concentration of cations in water that will react with a sodium soap to precipitate an insoluble residue. In general, hardness is a measure of the concentration of calcium and magnesium ions in water, and is expressed as mg/L calcium carbonate or equivalent.

Luminescent means emitting light, from a cause other than high temperature.

Lyophilized means freeze-dried under a vacuum, and is applied to the bacteria used in the Microtox test, as received from the supplier.

Monitoring is the routine (e.g., daily, weekly, monthly, quarterly) checking of quality, or collection and reporting of information. In the context of this report, it means either the periodic (routine) checking and measurement of certain biological or water-quality variables, or the collection and testing of samples of effluent, leachate, elutriate or marine/estuarine receiving water for toxicity.

Percentage (%) is a concentration expressed in parts per hundred parts. One percent represents one unit or part of substance (e.g., effluent, leachate, elutriate, or receiving water) diluted with water to a total of 100 parts. Concentrations can be prepared on a volume-to-volume or weight-to-weight basis, and are expressed as the percentage of test substance in the final solution.

pH is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions on a scale from 0 to 14, with 7 representing neutrality, numbers less than 7 signifying increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.

Precipitation means the formation of a solid (i.e., precipitate) which comes from a solution.

Pre-treatment means, in this report, treatment of a sample or diluting it, prior to testing its toxicity.

Salinity is the total amount of solid material, in grams, dissolved in 1 kg of aqueous solution. For seawater, salinity is determined after all carbonates have been converted to oxides, all bromide and iodide have been replaced by chloride, and all organic matter has been oxidized. Salinity can also be measured directly using a salinity/conductivity meter or other means (see APHA *et al.*, 1989). Salinity is reported as a percentage, to agree with Microtox manuals. The normal unit would be g/kg, or the approximate equivalent of that, parts per thousand (‰).

Surfactant is a surface-active substance (e.g., detergent) which, when added to a non-aqueous liquid, decreases surface tension and facilitates dispersion of materials in water.

Turbidity is the extent to which the clarity of water has been reduced by the presence of suspended or other matter that causes light to be scattered and absorbed rather than transmitted in straight lines through the sample. It is generally expressed in terms of Nephelometric Turbidity Units.

Terms for Test Substances

Blank is used interchangeably with the term control (*q.v.*), in this document.

Chemical is, in this report, any element, compound, formulation or mixture of a substance that might enter the aquatic environment through spillage, application, or discharge. Examples of chemicals that are applied to the environment are insecticides, herbicides, fungicides, sea lamprey larvicides, and agents for treating oil spills.

Control is a treatment in an investigation or study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific condition that is being studied. In an aquatic toxicity test, the control must duplicate all the conditions of the exposure treatment(s), but must contain no test substance. The control is used to determine the absence of measurable toxicity due to basic test conditions (e.g., quality of the dilution water, health of test organisms, or effects due to their handling).

Deionized water is water that has been purified to remove ions from solution by passing it through resin columns or a reverse osmosis system.

Diluent is the standard water used for dilution of test substance in the Microtox test; see also *dilution water*.

Dilution water is the water used to dilute a test substance in order to prepare different concentrations for a toxicity test. The standard dilution water used in the Microtox test is a specific formulation of saline water called *Diluent*.

Distilled water is water that has been passed through a distillation apparatus of borosilicate glass or other material, to remove impurities.

Effluent is any liquid waste (e.g., industrial, municipal) discharged to the aquatic environment.

Elutriate is an aqueous solution obtained after adding water to a solid substance (e.g., sediment, tailings, drilling mud, dredge spoil), shaking the mixture, then centrifuging or filtering it or decanting the supernatant.

Estuarine water is brackish seawater, residing in a coastal body of ocean water that is measurably diluted with fresh water derived from land drainage.

Pore Water is the water occupying space between sediment particles. The amount of pore water is expressed as a percentage of the wet sediment, by weight.

Leachate is water or wastewater that has percolated through a column of soil or solid waste within the environment.

Marine water is seawater residing in or obtained from the ocean, sea, or inshore location where there is no appreciable dilution by natural fresh water derived from land drainage.

Receiving water is natural surface water (e.g., in a river) that has received a discharged waste, or else is about to receive such a waste (e.g., it is just "upstream" or up-current from the discharge point). Further descriptive information must be provided to indicate which meaning is intended.

Reference toxicant is a standard chemical used to measure the sensitivity of the test organisms in order to establish confidence in the toxicity data obtained for a test substance. In most instances a toxicity test with a reference toxicant is performed to assess the sensitivity of the organisms at the time the test substance is evaluated, and the precision of results obtained by the laboratory for that chemical.

Sediment is a natural particulate substance that has been transported to, and deposited at the bottom of, a body of water. [In certain sections of this report, "sediment" is designated as a term of convenience which includes similar substances such as industrial and municipal sludges, and soils.]

Stock solution is a concentrated aqueous solution of the substance to be tested. Measured volumes of a stock solution are added to dilution water in order to prepare the required strengths of test solutions.

Substance is a particular kind of material having more or less uniform properties.

Upstream water is surface water (e.g., in a stream, river, lake, estuary, or marine waterbody) that is not influenced by the effluent (or other test substance), by virtue of being removed from it in a direction against the current or sufficiently far across the current.

Wastewater is a general term that includes effluents, leachates, and elutriates.

Toxicity Terms

Acute means within a short period in relation to the life span of the organism, and would be of the order of some minutes for bacteria, and usually ≤ 4 days for fish.

EC₅₀ is the median effective concentration, i.e., the concentration of substance in water that is estimated to cause a specified effect in 50% of the individuals exposed to that concentration. The effect could be lethal but is usually sublethal. *EC₅₀*, like *LC₅₀*, refers to a quantal effect since each exposed individual must be categorized as either showing the effect or not showing it. The effect must be specified, and often also the exposure time, for example "the

two-month EC_{50} for reproductive failure" or "the EC_{50} for avoidance reactions". The term does not apply to a percent reduction in some rate or process in an organism or a group of organisms; accordingly, this terminology should not be used in the Microtox test (see ICp).

Endpoint means the variables (i.e., time, reaction of the organisms, etc.) that indicate the termination of a test, and also means the measurement(s) or value(s) derived, that characterize the results of the test (lethal concentration, IC_{50} , etc.).

ICp is the inhibiting concentration for a (specified) percent effect.

It represents a point estimate of the concentration of test substance that would cause a designated percent impairment in a quantitative biological function such as light production by bacteria or growth of fish, relative to the control. This term should be used for any toxicological test which measures a change in rate, such as reproduction, growth or respiration. (The term *median effective concentration* (EC_{50}) is not appropriate in tests of this kind because it is limited to quantal measurements, i.e., an estimate that 50% of the individual organisms which were exposed to that concentration would show a particular effect, while the other 50% would not show the effect.)

LC₅₀ is the median lethal concentration, i.e., the concentration of substance in water that is estimated to be lethal to 50% of the test organisms exposed to that concentration. The LC_{50} and its 95% confidence limits are usually derived by statistical analysis of mortalities in several test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 7-d LC_{50}). This endpoint cannot be used in the Microtox test.

Lethal means causing death by direct action. Death is usually defined as the cessation of all visible signs of movement or other activity.

Static describes toxicity tests in which test solutions are not renewed during the test.

Sublethal means detrimental to a living organism, but below the level that directly causes death within the test period.

Toxicity is the inherent potential or capacity of a substance to cause adverse effects on living organisms. The effect could be lethal or sublethal.

Toxicity Identification Evaluation describes a systematic sample pre-treatment (e.g., pH adjustment, filtration, aeration) followed by tests for toxicity. This evaluation is used to identify the causative agent(s) that are primarily responsible for toxicity in a complex mixture. The toxicity test can be lethal or sublethal.

Toxicity test is a determination of the effect of a substance on a group of selected organisms, under defined conditions. An aquatic toxicity test usually measures either (a) the proportions of organisms affected (*quantal*), or (b) the degree of effect shown (*graded or quantitative*), after exposure to specific concentrations of chemical, wastewater, receiving water, or liquid derived from sediment or similar solid substance. The assay with luminescent bacteria must be considered a graded toxicity test since there is no measurement of the proportions of individual bacteria that are directly affected, but instead, overall measurements of the degrees of reduction in a physiological function, shown by groups of bacteria.

Acknowledgements

This report was co-authored by J.B. Sprague (Sprague Associates Ltd., Guelph, Ontario) and D.J. McLeay (McLeay Associates Ltd., West Vancouver, B.C.). It is largely based on pre-existing procedures of Microbics Corp. (see Introduction). Messrs. G.A. Sergy and R.P. Scroggins (Environmental Protection, C&P, Environment Canada) acted as Scientific Authorities and provided technical input and guidance throughout the work.

Members of the Inter-Governmental Aquatic Toxicity Group (IGATG, Appendix A) are thanked for their active participation in developing and reviewing this report. Special acknowledgement is made of the technical contributions from the IGATG Sub-Committee responsible for initial and final review: G. Joubert (Québec Ministère de l'Environnement, Ste-Foy, Que.); M. Korchinski (Alberta Energy Resources Conservation Board, Calgary, Alta.); A.A. Qureshi (Alberta Environmental Centre, Vegreville, Alta.); and IGATG members C. Blaise, B.J. Dutka, G. Elliott, and G.C. van Aggelen. The laboratory testing support of Environment Canada (Appendix B) is acknowledged. Mr. P.C. Thomas of the former Microbics Enterprises (Forest, Ont.) generously provided technical literature and loan of equipment for trial.

The following persons provided information and many useful comments on final or early drafts: C. Bastien (Québec Ministère de l'Environnement, Ste-Foy, Que.); Y. Bois (Technitrol-Eco, Pointe Claire, Que.); A.A. Bulich and M.W. Greene (Microbics Corp., Carlsbad, CA); J.E. Cairns (Dearborn Chemical Co. Ltd, Mississauga, Ont.); N.A. Casseri (OxyChem, Grand Island, NY); J. Coyle (U.S. Fish & Wildlife Service, Columbia, MO); K.G. Doe, M. Nicol and J.D.A. Vaughan (C&P, Dartmouth, N.S.); E. Dombroski (Alberta Environmental Centre, Vegreville, Alta.); J.I. Fujikawa (Alberta Environment, Lethbridge, Alta.); M.S. Henebry (Illinois Environmental Protection Agency, Springfield, IL); K. Ho (Univ. of Rhode Island, Narragansett, RI); R.A. Hoke (ASCI Corp., Duluth, MN); K.L.E. Kaiser and S. Skog (C&P, Burlington, Ont.); L. Kennedy (Massachusetts Environmental Quality Engineering, Westborough, MA); G. Kurz (Dept of Public Works, Chattanooga, TN); E. Lee (B.C. Research, Vancouver, B.C.); E.A. Power (E.V.S. Consultants, North Vancouver, B.C.); Y. Roy (Analex Inc.); B. Salahub (Chemical & Geological Laboratories Ltd., Edmonton, Alta.); J. St-Onge (Stone-Consolidated Inc., Grand'Mère, Que.); and S. Yee (C&P, North Vancouver, B.C.).

Photographs for front cover supplied by Dr. Christian Blaise, Ecotoxicology and Ecosystem Branch, Centre Saint-Laurent, Environment Canada.

Section 1

Introduction

1.1 Background

Aquatic toxicity tests are used within Canada and elsewhere to measure, predict, and control the discharge of substances that might be harmful to aquatic life.

Recognizing that no single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection, the Inter-Governmental Aquatic Toxicity Group (members in Appendix A) proposed the development and standardization of a set of single species aquatic toxicity tests that would be broadly acceptable, and would measure different toxic effects using organisms representing different trophic levels and taxonomic groups (Sergy, 1987). A toxicity test with luminescent bacteria was one of several "core" aquatic toxicity tests that were selected to help meet Environment Canada's testing requirements.¹

Universal procedures for an assay with luminescent bacteria are described in this report. Also presented are specific sets of test conditions and procedures, required or recommended when using the test for evaluating different types of substances (namely samples of chemical, effluent, leachate, elutriate, receiving water, sediment, or other solid substance) (see Figure 1). Those specific procedures and conditions, relevant to conducting the test and standardizing it, are delineated and, as

appropriate, discussed further in explanatory footnotes.

Although this assay can be generically described as a test of light production by a strain of bacteria, methods described herein are for the only test system commercially available within Canada at the time of this writing², the *Microtox*TM test. The test method described here is the exclusive property of Microbics Corp. of Carlsbad, California (*Microbics*).

Microbics provides explicit instructions for conducting the test, and this report does not replace those instructions. It is, rather, a summary of the instructions to facilitate orientation and guidance, and provide a supplement. The test procedures available from *Microbics* and from governmental or international agencies do not necessarily address all issues that are important in Canadian government aquatic toxicity laboratories. They do not necessarily cover methods for handling different kinds of samples, the question of pH adjustment, variations in methods associated with differing test objectives or types of samples, or how to deal with samples which contain appreciable solids or floating material. Existing methodology documents usually include procedures for testing samples of effluent or chemical, but sometimes provide only minimal guidance for testing samples of leachate, elutriate, receiving water and sediment or similar solid.

1 Methods for five tests with fish and crustaceans have already been published as the result of the IGATG proposal (Environment Canada, 1990a; 1990b; 1990c; 1992a; 1992b).

2 A similar, competitive method (*Lumistox*TM) and products are now being marketed in Canada by InterSciences Inc. (169 Idema Rd., Markham, Ont. L3R 1A9; P.O. Box 1311, Edmonton, Alta. T5J 2M8; phone no. 1-800-661-6431

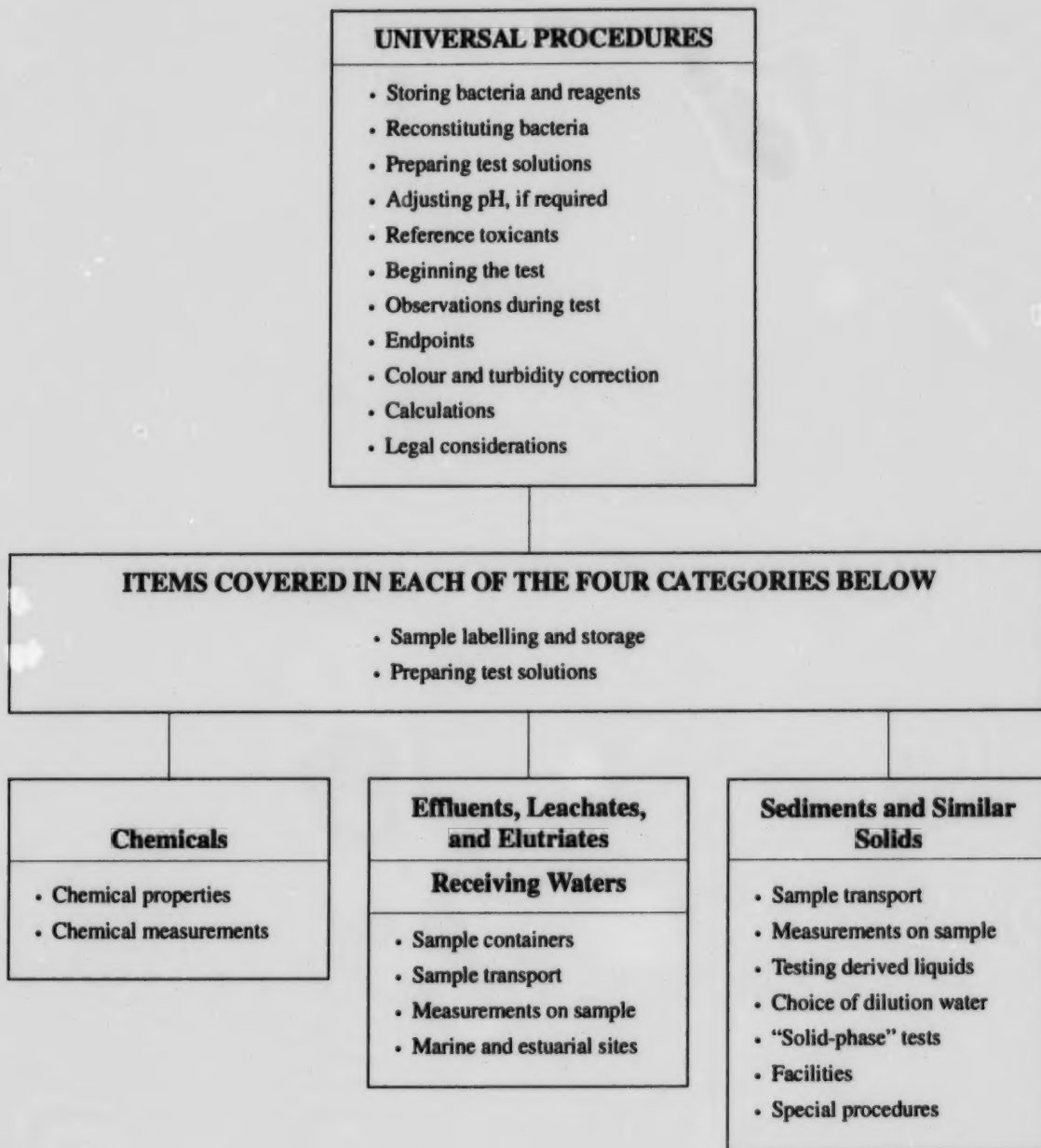


Figure 1 **Diagram of Approach Taken in Delineating Test Conditions and Procedures Appropriate for Various Types of Substances**

In describing these procedures, an attempt was made to balance scientific, practical, and financial considerations, and to ensure that the results would be accurate and precise enough for the majority of situations in which they will be applied. The authors assume that the user has a certain degree of familiarity with aquatic toxicity tests. Explicit instructions on every detail are not provided in this report, since those can be obtained from the Microtox manuals.

1.2 The Test and Test Species

A specific strain of the marine bacterium *Photobacterium phosphoreum*³ is used in this test to determine the toxicity of samples. This bacterium emits light as the result of normal metabolic processes, and the light is measured with a standard photodetection device under specific conditions. Reduction of light at 5, 15, or 30 minutes is taken as a measure of toxicity.

The Microtox toxicity test was developed commercially and first offered for sale in 1978. It is now marketed and used around the world, and there is extensive scientific literature on the test and the results of using it (Bulich, 1986; and Microbics, 1989a). A toxicological data bank of appreciable magnitude has been assembled for the Microtox tests by Canadian scientists Kaiser and Ribo (1988). The bacterial bioluminescence test is not limited to aquatic pollutants. For example, it is being

considered by the United States Pharmacopeia for testing materials used as containers for drugs and medical devices (USP, 1989).

The background of previous toxicity data for this test, its demonstrated sensitivity to aquatic contaminants, and its widespread availability as a standardized procedure make it a logical choice as one tool for tests in Canadian laboratories.⁴ It has been compared with 50 other microscale tests (Munkittrick and Power, 1989), and described as "very useful as field microassay for screening purposes", with a high rating for "environmental relevance" (defined as similarity to whole-organism effects on fish and crustaceans). At least one province has adopted a standard Microtox procedure (BNQ, 1987), and other provinces have informal documents outlining procedures (Subsection 1.2.1). Microtox has been specified for three-times-a-week effluent monitoring at certain pulp mills in British Columbia.

The test is particularly useful for exploration or monitoring because it is rapid, simple, uses small samples, and is inexpensive once the photometer (*Analyzer*)⁵ has been purchased. It is suitable for assessing the short-term toxicity of industrial or municipal effluents, leachates, elutriates, mixing zones of surface waters, chemicals, toxicants released from sediments or soils, and in fact, materials that enter water from a variety of sources. Microtox can also directly test

3 There are many strains of this bacterium, and at least some of them are known to differ in their patterns of sensitivity to toxicants. The strain designated by *Microbics* for use in their test is "NRRL B-11177" deposited with the Northern Regional Research Laboratory in Peoria, Illinois, U.S.A.

4 Use of the test does not indicate endorsement by Environment Canada or any of its laboratories.

5 This report has adopted most of the terminology used by *Microbics* for reagents, apparatus, and processes, to allow the user to work more easily between this report and the *Microbics* manuals. Some terms can have different connotations in ordinary usage. To facilitate use in this report, specific terminology of *Microbics* is printed in italics with the initial letter(s) in upper case.

samples of sediment or other semi-solid substance such as municipal or industrial sludge, and has been recommended for testing soils at contaminated waste sites (U.S. EPA, 1987; 1989c). It could be used to assess the progress of detoxification or biodegradation of toxic substances.

The test is convenient since there is no need to maintain a living culture; bacteria are purchased in a freeze-dried state and can be stored for months as if they were a chemical reagent. This characteristic makes Microtox convenient for on-site comparisons of toxicity over time for a given industry or location. The rapidity of testing and ability to carry out many assays with little additional expense facilitates broad screening programs for toxicity of effluents or chemicals, toxic components in a complex waste, or other sizeable investigations. For example, it has been used for surveys of large sections of the St. Lawrence River (Kaiser *et al.*, 1988a).

The sensitivity of this test is, in general, similar to that of acute lethality tests using fish (reviewed by Munkittrick and Power, 1989; Munkittrick *et al.*, 1991). Compared to lethality tests that used fathead minnows, trout, and *Daphnia*, Microtox was about as sensitive to pure organic compounds, municipal wastes, and the more toxic industrial effluents, but was often less sensitive to inorganic toxicants and pesticides (Munkittrick and Power, 1989; Munkittrick *et al.* 1991).

Microtox can be appreciably more or less sensitive than a particular species of fish or other multi-cellular organism to any given toxic substance, with hundred-fold differences one way or the other in some cases (Munkittrick *et al.*, 1991). This is not necessarily a disadvantage since micro-organisms are sometimes among the

most sensitive species in aquatic ecosystems, and it has been recommended that they should always be included in toxicity evaluations (Slooff *et al.*, 1983). This recommendation is in keeping with the excellent principle of using a battery of toxicity tests. For some toxicants, an invertebrate or micro-organism might be most sensitive, and for other toxicants a fish or plant might be most sensitive. Therefore, to protect an aquatic ecosystem, toxicity information should be available for a variety of organisms. The corollary is that one type of organism should not be expected to predict sensitivity of another type of organism. It should not be expected that Microtox will necessarily predict the results of a test with trout, nor that the trout test will predict the result from Microtox. Rather, any such lack of correlation should be regarded as further evidence of the desirability of testing a variety of organisms.

Photobacterium phosphoreum is a marine organism, and the Microtox test is normally carried out at 2% salinity by adding a salinity-adjusting solution (or NaCl) to the sample and using a dilution water (*Diluent*) that has salinity of 2%. Light production of this bacterium is as high at 2% salinity as in full-strength seawater of about 3.5%, but varies somewhat within that range, with a peak at 2.7% (Krebs, 1983). Accordingly, samples of full-strength seawater may be tested if desired, but it is important to carry out the test at $\geq 2\%$ salinity.

It is conceivable that the toxicity of a given substance might be different in fresh and salt water, and that Microtox results might therefore be less suitable for protecting fresh waters. Most of the common toxicants, however, are of similar toxicity to marine and freshwater organisms when each is tested in its own medium (Sprague, 1985). Experience has shown that Microtox

generally gives toxicity results similar to those for acute lethality to freshwater organisms (Blaise *et al.*, 1987; Munkittrick *et al.*, 1991), and the test is accordingly suitable as one tool for investigating freshwater situations. An option for adjusting osmotic strength of solutions with sucrose instead of salts might have relevance to freshwater situations (see Subsection 4.8.4).

Within Canada, the source of procedural manuals as well as the specific strain of bacterium, equipment, and supplies for the Microtox test is *Microbics*.⁶ Earlier manuals prepared by *Microbics* are included in the reference list (Microbics, 1988a; 1988b; 1989b). *Microbics* recently produced a comprehensive and up-to-date manual (Microbics, 1992). An older "operating manual" (Beckman, 1982) provides useful detail on some aspects of testing. Various "Microtox application notes" are listed in the reference section under *Microbics* (1983), and some particular applications are included in general manuals (Microbics, 1989b). These deal with specific topics such as a rapid screening method, complex effluents, and groundwater. A training video is available from *Microbics*, and provides useful orientation for conducting the assay. A bibliography lists scientific projects that have used or evaluated the Microtox test (Microbics, 1989a).

Quebec has an official Microtox method (BNQ, 1987). Methods guides have been produced by two branches of the Alberta government, i.e., the Energy Resource Conservation Board (Alberta, 1986), and Alberta Environment (Alberta, 1987). In Alberta, Microtox is routinely used to test extracts of hydrocarbons, drilling fluids, soils, and sediments, as well as chemicals and water. An informal "Western Canada Microtox Users Committee" has conducted round-robin testing and facilitated standard approaches. The B.C. Ministry of Environment has an in-house guidance document for tests. A procedural guide has also been compiled by Environment Canada (Dutka, 1988). A brief description of a standard operating procedure has been prepared by a testing centre of the U.S. Environmental Protection Agency (U.S. EPA, 1989b). In Germany, a draft standard method (DIN, 1989) gives generic instructions for a photometer and for making up solutions. The German instructions parallel the Microtox method without naming it or Microbics Corporation, but mention that the freeze-dried bacteria are commercially available. All of these documents have been used in preparing this report and, in general, all of them describe the same method.

6 The toll-free phone and fax numbers to reach *Microbics* from Canada are: 1-800-245-7629 and 1-800-528-5558, respectively. Microbics Corporation, 2232 Rutherford Road, Carlsbad, California 92008, U.S.A. Mention of commercial products and their suppliers does not imply endorsement by Environment Canada or any of its laboratories. This information is provided for convenience of the reader.

Section 2

Test Organisms

2.1 Species

Organisms used in this test come from a standardized culture, and belong to a particular strain of *Photobacterium phosphoreum* (a strain designated NRRL B-11177). This is a bacterium which normally lives in the oceans, and produces blue-green light by enzymatic reactions, on a continual basis if sufficient oxygen is available.

2.2 Source and Holding

The standard culture of organisms is obtained from *Microbics*. According to *Microbics*, the bacterium is cultured as a genetically uniform strain, then harvested during the exponential phase of growth and lyophilized (i.e., freeze-dried under vacuum). Bacteria are purchased in that

state, in small lots in sealed containers, each lot suitable for at least two hours of testing after they have been reconstituted to an active state.⁷

The container of lyophilized *Bacterial Reagent* is said to be stable for one year when kept in a freezer at -20°C (*Microbics*, 1989b). Storage can be "extended somewhat" at -40°C, while storage in a refrigerator at 4°C "greatly shortens shelf-life" (*Microbics*, 1992). Storage temperature should be constant, and therefore self-defrosting or "frost-free" refrigerators and freezers should not be used.

The bacteria are brought back to an active, living state (*Reconstituted Reagent*) by adding liquid (*Reconstitution Solution* or *Recon*) and bringing them to a temperature of 5°C.

7 See Subsection 4.3.1 for further information on useful working time for the *Reconstituted Bacteria*.

Section 3

Test System

3.1 Principles of Test and Variability

Subsamples of *Reconstituted Bacteria* are exposed to concentrations of the sample. About a million bacteria are introduced into each test vessel. Any toxic action of substances in the sample is presumed to affect metabolic processes of the bacteria, and bioluminescence is inhibited in proportion to the metabolic effect. That inhibition is measured and expressed as the IC₅₀ (concentration causing 50% inhibition⁸) after specific periods of exposure (some or all of 5, 15, and 30 minutes).

3.1.1 Limits and Reproducibility

All manipulations of the sample and the bacteria are manual, and depend on the skill of the operator in reconstituting the bacteria, handling the micropipettor, mixing solutions, etc. Variability in volumes transferred by a good operator might contribute about 1% uncertainty in light readings. Geometrical variations in cuvettes contribute another 1% (Beckman, 1982).

Tests by the Quebec Ministry of Environment (BNQ, 1987) involving 236 results indicated that 17% and 83% inhibition of light production represented the minimum and maximum values that could be quantified with statistical significance. The

detection limit in the same work was 12% inhibition.

Variability of Microtox is low compared to other tests of aquatic toxicity. In a series of 81 tests with a reference toxicant (sodium lauryl sulphate), the overall coefficient of variation was 18% (Bulich *et al.*, 1981).⁹ Within the three lots of bacteria used in the tests, the individual coefficients of variation were from 6 to 10%. Although three people carried out the tests on three different *Analyzers*, this did not lead to significant differences in results. An overall mean deviation of replicates from the mean IC₅₀ for eight organic chemicals was 10% (Curtis *et al.*, 1982). Tests done for the Canadian Petroleum Association by three laboratories showed an average coefficient of variation of 11% (Stroscher, 1984). Munkittrick and Power (1989) summarize these comparisons and others by listing average coefficients of variation that range from 2 to 30%, except for metal tests which had an average coefficient of 60%.

3.1.2 Interference and Other Limitations

High colour of a sample, particularly red or brown, can interfere with light transmission and therefore with toxicity measurements. This could be important for some effluents such as those from pulp mills. A correction for this may be made by calculation after

8 Throughout the literature from *Microbics*, generally in U.S. scientific literature, and often in Canadian work, the IC₅₀ has been wrongly called the EC₅₀ (see *Terminology*). This will no doubt be corrected in the future, as the correct terminology has been recognized by ASTM and is being promulgated by that organization for appropriate tests.

9 C. Bastien of the Quebec Ministry of Environment reports similar coefficients of variation of 15 to 20% during four years of testing with this same reference toxicant.

performing an additional test using a *Colour Correction Cuvette*, as described in Section 4.9.

Turbidity resulting from suspended solids can also reduce light transmission and increase the toxicity estimate. The colour-correction method previously mentioned may be used with dark, optically absorbing particles, but is not effective if used with white, optically reflecting particles. Centrifugation will sometimes remedy the turbidity problem (see Subsection 4.1.1).

The highest concentration of sample tested in the standard Microtox technique is 45% (see Subsection 4.2.2). If the sample is weakly toxic, an investigator might wish to test a nearly full-strength sample. This can be done by using the special technique described in Subsection 4.8.3.

Exposure times are somewhat arbitrary as in all toxicity tests, and appropriate ones must be adopted by judgement. Most Microtox tests are completed in 15 minutes. Phenol completes its action within five minutes, so the best measure of its toxicity is the 5-min IC₅₀. For bivalent metals, the light output might still be decreasing after 15 minutes, and the IC₅₀ at 30 minutes or longer will be most sensitive. For unknown samples, therefore, readings should be taken at several standard times, and the most appropriate one(s) chosen. The best exposure would be the one when light inhibition levelled off, or just after that, i.e., allowing just enough time for the toxicant to exert its maximum action.

3.2 Facilities

The test can be conducted in a normal, clean laboratory with standard lighting. The need for any special facilities would be governed by the degree of hazard associated with the

samples or chemicals that were to be tested, and by the risk of sample contamination.

3.2.1 Photometer

Light production could be measured using a variety of photometers, such as a standard photometer connected to a water bath (Dearborn, 1986), or an adenosine triphosphate (ATP) photometer as evaluated by Awong *et al.* (1989). The *Microbics Toxicity Analyzers* Model 2055 and 500 are, however, conveniently designed to carry out the test with dispatch under controlled conditions. Use of those machines is described herein, and their general appearance is shown in Figure 2 (Model 2055 Analyzer) and Figure 4 in Section 5 (Model 500 Analyzer). Methods in general, as well as particular methods for the Model 2055 Analyzer are described in Section 4. Because many of the operations are performed manually with the Model 2055, Section 4 provides an awareness of the steps in an analysis. For the newer Model 500, many of the steps are carried out automatically. The methods for this machine are described in Section 5.

Using the *Toxicity Analyzer* Model 2055, light output of the bacteria is measured by a photomultiplier tube which has two ranges, 1X (no enhancement) or 10X (tenfold enhancement). The *Analyzer* is more than a photometer since it holds all the cuvettes for a test and keeps the contents at a pre-selected temperature. Circuits are built in to allow temperature readout from areas that contain test solutions and bacteria.

The layout of the top working surface of the Model 2055 *Toxicity Analyzer*, with which an operator must be familiar, is shown in the lower part of Figure 2. The fifteen numbered circles A1 to C5 are the incubator wells which can incubate cuvettes of sample and control solutions at $15 \pm 0.3^\circ\text{C}$. The

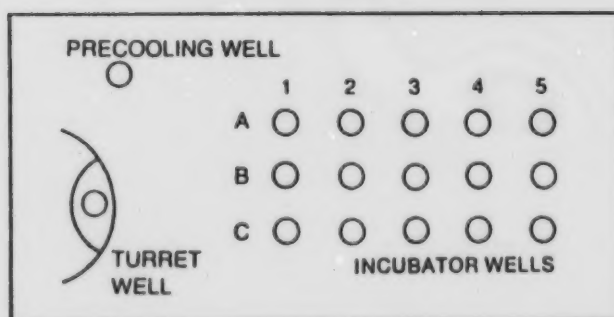
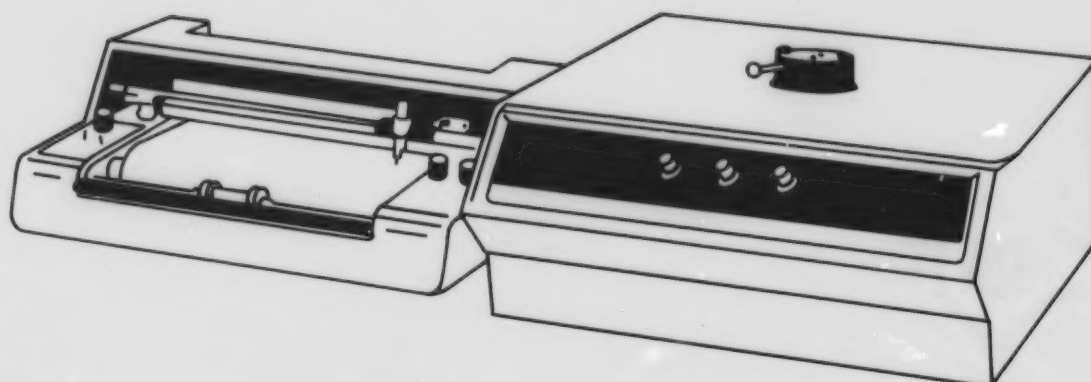


Figure 2 Appearance of the *Microbics Toxicity Analyzer Model 2055 and Chart Recorder*

The upper part of the figure shows the *Toxicity Analyzer* on the right, with the "turret" protruding from the top, and a chart recorder on the left. The lower part of the figure shows the wells used to contain the test cuvettes, on the top surface of the *Toxicity Analyzer*.

Precooling Well holds the *Reconstitution Solution* and bacteria at 3 to 5°C. The *Turret Well* is where the reading of light production takes place; cuvettes must be cycled in turn to that well.

3.2.2 Other Equipment

Other equipment necessary to perform these tests include:

- (a) **Freezer or refrigerator** for storage of reagents. A freezer should maintain its contents at -20°C. A refrigerator should be capable of regulation between 2°C and 8°C. Neither machine should be self-defrosting.
- (b) **pH meter.**
- (c) **Count-down timer or stopwatch.** (Alternatively, the chart recorder may be used as a timer – an easier and more fail-safe procedure.)
- (d) **Chart recorder or computer-based recording system.** Light emission values may be read from the *Toxicity Analyzer* and written down, but a recorder is advantageous. A suitable chart recorder would be a 25-cm, single-pen, manual-lift model.¹⁰ *Microbics* offers computer software, version 6.0, which allows a personal computer to collect data directly. The software and computer replace the recorder with the newer Model 500 *Analyzer* (see Section 5), and with Model 2055 *Analyzers* which are equipped with the necessary interface.
- (e) **Calculator or computer.** Results from the test can be conveniently processed in

BASIC on an IBM-compatible personal computer. The software is available from *Microbics*. Data can also be processed with a Sharp Model EL-5150 programmable calculator, a device which is no longer sold.

3.2.3 Supplies

Supplies required include:

- (a) Volumetric borosilicate glassware for processing small samples (acid-washed).
- (b) **Disposable glass incubation cuvettes**, 12 × 50 mm. These are the containers used as test vessels for the various dilutions in the *Analyzer*.
- (c) **Colour Correction Cuvette.** A reusable double cuvette used for correcting readings with coloured or turbid samples.
- (d) **Micropipettors** of 10, 200 or 250, 500, and if desired, 50 and 1000 µL, with disposable plastic tips. The micropipettors are also used for mixing liquids in cuvettes since they aspirate as well as dispense accurately.

3.2.4 Organisms and Reagents

Organisms and reagents required include:

- (a) **Supply of bacteria** (*Microtox Reagent* or more recently *Bacterial Reagent*). *Photobacterium phosphoreum* are obtained from *Microbics* as small, sealed containers of about 100 million lyophilized organisms. Storage should be in a freezer or a refrigerator, the former being suitable for at least a year of storage, the latter for an unspecified but much shorter time. After

¹⁰ *Microbics* offers a compatible chart recorder, Part No. 686008, and previously recommended a Beckman Model 2055 as a match for their Model 2055 *Analyzer*.

reactivation of the bacteria, *Microbics* uses the name *Reconstituted Reagent*.

- (b) **Reconstitution Solution (Recon)** is distilled water, free of toxic material (*Microbics*, 1989b); used to reconstitute the bacteria to their active living state at the start of a test. It is supplied in sealed containers by *Microbics*, must not be frozen, and may be stored for a year at a temperature between 2 and 8°C, or at room temperature for an unspecified period of time.
- (c) **Diluent** is used to dilute the samples to desired concentrations; it is supplied by *Microbics*, and contains 2% sodium chloride in purified water; storage requirements are the same as for *Recon*.

A special *Solid-Phase Diluent* is used in the "solid-phase" test (see Subsection 9.3.1).

- (d) **Microtox Osmotic Adjustment Solution (MOAS)**. A 22% solution of sodium chloride for increasing salinity of samples to the desired level. The assay is normally run at 2% salinity, so adjusting a freshwater sample is done by adding one part of *MOAS* to 10 parts of *sample*. Storage requirements are the same as for *Recon*.
- (e) **Sodium chloride** (NaCl, Analytical Grade, 99%). This may be used, if desired, for adjusting a sample to the desired salinity for testing.

Section 4

Universal Test Procedures

Procedures described in this section are for use with the Microtox Analyzer Model 2055. Many of the concepts and general procedures for the Model 2055 also apply to tests with the newer Model 500 Analyzer, made available by Microbics in early 1989. Details of procedures vary, however, and many of the steps are automated in the newer Model 500 (see Section 5). The procedures in this section apply to all the tests described in Sections 6, 7, 8, and 9 for particular chemicals, wastewaters, and sediments or similar solids. All aspects of the test system described in the preceding section must be incorporated into these universal test procedures. The summary checklist of recommended conditions and procedures in Table 1 includes not only universal procedures but also those for specific types of test materials.

A brief summary of the procedure follows. A series of dilutions of the sample plus control(s) is tested. Light outputs of the cuvettes of *Reconstituted Bacteria* are measured before the sample (test solution) is added, then again after 5 and 15 minutes of exposure, and perhaps after 30 minutes or longer if there are slow-acting toxic agents. The readings are corrected according to the change in the control(s) (nontoxic *Diluent* only), to allow for drifts in light output over time, and small effects from dilution of the bacteria when sample is added. A dose-effect curve is analyzed, and the concentration causing 50% inhibition of light production is estimated mathematically.

The following outline is organized by category, and does not necessarily represent

the most efficient chronological sequence for testing. *Microbics* instructions give guidance on sequence.

4.1 Interfering Characteristics of Sample

4.1.1 Colour, Turbidity, and Floatables

Check for colour or turbidity of the sample to be tested, as either of these could affect light measurements. It is difficult to specify numerical guidelines for troublesome levels of such variable characteristics as colour or turbidity, but the optional Microtox procedure to correct for these qualities is simple enough to carry out (see Section 4.9) and should be used if the investigator has any doubts about light transmission.

If the turbidity is light-reflecting, the correction procedure given in Section 4.9 is not suitable. The preferred option is to remove the suspended matter causing the turbidity, and test for the residual toxicity in the liquid part of the sample. Filtration might be satisfactory but the particular kind of filters to be used should be checked by a Microtox test on *Diluent* run through the filter. Some filter papers and apparatus can add measurable toxicity, according to Microbics (1988b), sometimes because of wetting agents added to the filter. A filter paper might also sorb toxic substances and remove them from the sample filtrate. Suspended matter can be reduced by centrifugation, or by a few hours of settling.

There is no standard recommendation for dealing with the problem of material floating on top of the sample. It is not possible to put

Table 1 Checklist of Recommended Test Conditions and Procedures**UNIVERSAL**

Test type	- static, 5 and 15 min duration, to 30 or 60 min if necessary
Species	- <i>Photobacterium phosphoreum</i> , strain NRRL B-11177
Facilities	- Microbics photometer, either Model 2055 or automated Model 500
Control/dilution water	- standard pure water with 2% NaCl (<i>Diluent</i>); optional: sucrose instead of NaCl for increased sensitivity to ammonia and some metals
Temperature	- $15 \pm 0.3^{\circ}\text{C}$
pH	- no adjustment if pH of sample is within the range 6.0 to 8.5; adjustment optional outside that range, depending on purpose
Colour, solids, or floatables	- use colour-correction technique for high colour and/or dark solids; remove light-coloured solids; for floatables, test underlying liquid
Oxygen/aeration	- normally no aeration of sample or prepared test concentrations; if dissolved oxygen is <40% or >100% saturation, it is optional to pre-aerate the sample or all test solutions for ≤ 20 min
Preparing test solutions	- highest concentration normally 45% with lower concentrations in two-fold dilutions; option for testing nearly full-strength sample, or other concentrations in logarithmic series
Observations	- light production initially, and at 5 and 15 min after introduction of sample; sometimes after 30 min or longer exposures
Endpoints	- IC50s at 5 and 15 min, and any other appropriate times
Reference toxicant	- choice of toxicants, tested at monthly intervals and with each new batch of test organisms; phenol, zinc, potassium dichromate, or sodium lauryl sulphate recommended
Test validity	- valid numerical estimate of ICp should be based on concentrations showing light inhibition both greater than, and less than, the inhibition at the ICp

Table 1 Checklist of Recommended Test Conditions and Procedures (Cont.)

CHEMICALS

- | | |
|---------------|---|
| Solvents | - these or other solubilizing aids (e.g., dispersants) used only in special cases |
| Concentration | - measure concentration in stock solution (desirable, not mandatory) |

EFFLUENTS, LEACHATES, AND ELUTRIATES

- | | |
|------------------------|---|
| Transport/storage | - if $>7^{\circ}\text{C}$, cool to 1 to 7°C (ice or frozen gel packs); transport and storage in dark at 1 to 7°C (preferably $4 \pm 2^{\circ}\text{C}$); store at $4 \pm 2^{\circ}\text{C}$ in the dark; sample must not freeze; test should start within 24 h, and must start within 72 h of sampling/extraction |
| Control/dilution water | - normal <i>Diluent</i> as in UNIVERSAL; clean seawater is option for effluents that will discharge to marine location, or for elutriate that utilized seawater |

RECEIVING WATERS

- | | |
|------------------------|--|
| Transport/storage | - as for effluents and leachates |
| Control/dilution water | - for fresh surface waters, as in UNIVERSAL; if sample is marine, adjust <i>Diluent</i> salinity or use clean seawater; if sample is estuarial, adjust <i>Diluent</i> to sample salinity if above 2‰ |

SEDIMENTS OR OTHER SOLIDS

- | | |
|--------------------------|--|
| Transport/storage | - temperatures as for effluents and leachates; test should start within two weeks and must start within six weeks |
| Preparing test substance | - centrifuge to separate pore water from solids; if desired, test pore water or aqueous extract as for effluent |
| Reference sediment | - parallel test with clean sediment of similar physicochemical properties |
| Facilities | - Model 500 photometer, or Model 2055 with computer interface |
| Control/dilution water | - standard <i>Solid-Phase Diluent</i> to test the sediment or other solid substance |
| Observations | - as UNIVERSAL, except that exposure is for 25 minutes and light emission of control also serves as "initial value" for all concentrations |
| Endpoints | - as UNIVERSAL, IC_{50} calculated by the solid-phase program of <i>Microbics</i> |

any mixing device into or onto the cuvettes during the test. Attempts to homogenize floating liquids, to last for the duration of the test, could cause suspended droplets that would interfere with light transmission. The matter is left to the judgement of the investigator, and depends on the type of material. Options would be a test of the underlying liquid or an attempt at homogenization, and the former is recommended.

4.1.2 pH

Toxicity tests should normally be carried out without adjustment of pH.¹¹ The pH of the sample should, however, be measured before testing it. If the sample is likely to cause the pH of any test solution to be outside of the

range 6.0 to 8.5¹², and/or it is desired to assess toxic chemicals, rather than the effect of pH itself or the modifying effect of pH on toxicity of substances in the sample, then the pH of an aliquot of the sample should be adjusted before using it.¹³ Alternatively, a second (pH-adjusted) test could be conducted concurrently with the unadjusted one.

For an adjusted test, the initial pH of the sample may, depending on objectives, be adjusted to within ± 0.5 pH units of that of the *Diluent*, or to within 0.5 units of the pH of natural seawater. Another acceptable approach for an adjusted test is to change the pH of the sample upwards to the range pH 6.0 to 6.5 (if the sample has pH <6.0), or

-
- 11 A justification for not changing the pH of the sample or solution is that pH can have a strong influence on the toxicity of a substance being tested, judging by results for testing fish and invertebrates. Toxicity might decrease (e.g., for ammonia if pH moves from alkaline to neutral), or might increase (e.g., for zinc moving from either acid or alkaline water to neutral). For the (generally) low concentrations of waste found in receiving water after dilution, any change in pH caused by the test substance, with concomitant modification of toxicity, could be accepted as "part of the pollution package". That leads to the rationale that the pH should not be adjusted in tests.
 - 12 Microbics (1988a) indicates no loss of light production caused by pH in the range 6.3 to 7.8. That range has been widened in this report for two reasons. First, Krebs (1983) shows a graph with light production affected no more than $\pm 5\%$ over the pH range 5.5 to 9.0. Second, *Photobacterium phosphoreum* is a marine organism, and the pH of the oceans has an average close to 8.1 (Thurman, 1975), frequently reaches pH 8.3, and has a normal range of 7.5 to 8.5, whether it is brackish or full strength. Since Environment Canada laboratories undoubtedly test samples of seawater that have received wastes, it is necessary to have an upper limit for testing that is pH 8.3 or higher, since that might be natural in the samples. The upper limit has been set slightly higher than the pH 8.3 in this report, with a recommended range of 6.0 to 8.5, selected on the basis of being 0.5 units within the range of little effect found by Krebs (1983). There might sometimes be reasons for adjusting pH within the recommended range for toxicological reasons described in this section.
 - 13 The rationale for making these adjustments does not contradict the previous rationale of accepting divergent pH as "part of the pollution package", but depends on the purpose of the test. Some chemicals and wastewaters will create levels of pH that have direct sublethal or lethal effects, especially in monitoring or compliance tests with full-strength effluent. An investigator might not be primarily interested in whether extreme pH is toxic, because such a pH might be unlikely after even moderate dilution in receiving water. If pH *per se* were of primary interest, it could be economically assessed by physicochemical measurements. An investigator would often wish to know if toxic substances were present in a wastewater, and detecting them would require elimination of any masking by toxic action of pH. That rationale leads to the use of pH-adjusted samples, in a parallel manner to the standardization of temperature and salinity at favourable levels when testing for toxic substances.

downwards to pH 8.0 to 8.5 (if the sample has pH >8.5).¹⁴ Solutions of HCl or NaOH at strengths $\leq 1 N$ should normally be used for all pH adjustments. Some situations (e.g., effluents with highly-buffered pH) might require higher strengths of acid or base, to avoid large changes in volume of the adjusted sample.

Adjustment of pH can cause precipitation of dissolved solids which could influence the readings of light in the tests; such precipitation should be watched for, and attempts made to deal with it if necessary (see Subsection 4.1.1).

Abernethy and Westlake (1989) provide useful guidelines for adjusting pH. Aliquots of samples receiving pH-adjustment should be allowed to equilibrate after each incremental addition of acid or base. The amount of time required for equilibration will depend on the buffering capacity of the sample. For effluent samples, a period of 30 to 60 minutes is recommended for pH adjustment (Abernethy and Westlake, 1989).

If the purpose of the toxicity test is to gain an understanding of the nature of the toxicants in an effluent, leachate, elutriate, or receiving-water sample, then pH adjustment is frequently used as one of several techniques (e.g., oxidation, filtration, air stripping, addition of chelating agent, etc.) for characterizing sample toxicity. Mount and Anderson-Carnahan (1988) list pH adjustment as one of nine Toxicity Identification Evaluation (TIE) techniques which, when performed with an acutely toxic aqueous sample, provide the investigator with a useful method for assessing the physicochemical nature of the toxicant(s) and susceptibility to detoxification.

4.1.3 Dissolved Oxygen and Aeration

Normally there is no aeration of samples or of a prepared test solution. This is not considered necessary for the Microtox test.¹⁵ Aeration is an option, however.¹⁶ If (and only if) the measured dissolved oxygen is <40% or >100% of air saturation in the sample or in an aliquot of a test solution when it has been made up to start the test, the sample or all solutions¹⁷ could be aerated

- 14 Addition of acid or base to an undiluted sample of effluent, leachate, or elutriate can significantly alter the ionized/non-ionized form of some toxicants (e.g., ammonia, resin acids, zinc) and can destroy the integrity of the test sample. If it is decided to adjust pH, it should be carefully done to just inside the limit specified, in this case 6.0 to 8.5, or within 0.5 units of the pH of *Diluent* or seawater. Overshoot should be avoided, especially "titrating through" pH 7.0 from either direction.
- 15 Several Canadian laboratories and Microbics report that low oxygen, at least any levels >1 mg/L, do not appear to affect the results of Microtox tests. Normal handling of samples, and standard dilution by one half with aerated solutions, is generally considered to provide adequate oxygen for the test, even if the sample is deficient.
- 16 Many of the biological test methods published by Environment Canada (e.g., 1990a; 1990b; 1990c; 1992a; 1992b) require aeration if there is low oxygen or supersaturation, and the option shown here is similar to the procedure that is a requirement in those tests. The option allows a laboratory to carry out the Microtox test with procedures that are as similar as possible to those which are used in other tests, if that is desired. For example, aeration can strip volatile chemicals from solution, or increase their rate of oxidation and degradation, changing the toxicity. If that were being done in a test with, say, rainbow trout, an investigator might wish to carry out the Microtox test in parallel fashion. Alternatively, two Microtox tests might be performed, with and without pre-aeration, to determine the effect of that treatment.
- 17 In the Microtox test, there would usually only be one test solution which could be aerated, and that would be the highest concentration intended for addition to a cuvette, or the sample itself. Lower concentrations would be obtained by dilutions in the cuvettes.

before starting ("pre-aeration"). For this purpose, oil-free compressed air should be dispensed through a disposable glass pipette, at a minimal rate for effective aeration of the particular vessel and volume of fluid being used. Duration of pre-aeration should be the lesser of 20 minutes and attaining 40% saturation (or 100% saturation, if supersaturation is evident) in the sample or highest test concentration. Any pre-aeration of test solutions or sample must be reported (Section 10).

4.2 Preparing for the Test

4.2.1 Setting up the Toxicity Analyzer

- (a) Check that the temperatures of the *Turret Well* and *Incubator* region are at $15 \pm 0.3^\circ\text{C}$. If not, adjust with the "TEMP SET" dial.
- (b) Set the photodetector in the 10X (enhancement) range. Set the SPAN (sensitivity) dial to maximum.
- (c) Confirm that the high voltage (HV) switch is on. It should be on for a few minutes before step (d).
- (d) Confirm that the photodetector output is 000, with the minus sign flashing. If not, set it at 000 with the ZERO dial.
- (e) Perform a calibration check. Set the SPAN at 4, then press the CAL CHECK button which turns on a tiny standard light in the *Turret Well*. The display should be in the range 80 to 120, and if so, testing can begin with the dials remaining in their existing positions.
- (f) Turn on the recorder with chart speed of 1 cm/min. Adjust pen to zero, and check that the photomultiplier reading of the *Toxicity Analyzer* remains at 000. The

recorder can be turned off until the appropriate time.

- (g) Put new cuvettes in each of the wells that will be used. All wells should contain cuvettes; those from a previous test can be left in wells not required for the current test.
- (h) If any of the above readings fall outside the specified range, consult the Microtox manual for remedial action.

4.2.2 Preparing the Test Solutions

Most of the vessels and measuring tools used in the test are disposable and are assumed to be clean and ready for use when obtained (e.g., cuvettes and tips for pipettors). Some additional measurement devices, stirring equipment, etc. might be required for handling the sample, and they must be thoroughly cleaned and rinsed in accordance with standard operational procedures. Washing of glassware has been known to add toxicity in the Microtox test, so rinsing must be thorough and it is recommended that a direct check be made for effect of any washing technique on results of the test.

For tests intended to estimate an IC₅₀, at least four test concentrations plus a control solution (100% *Diluent*) are to be prepared. Four concentrations must be considered a minimum with which to generate an adequate dose-response relationship, especially if the general degree of toxicity of the sample is unknown. BNQ (1987) calls for five concentrations. Six concentrations in a suitable series would usually be advantageous in routine use, and could easily be accommodated in the cuvette wells of the standard *Analyzer*. Use of six concentrations and a control is common practice at Canadian laboratories, and is recommended. As in other toxicity tests, the standard series of concentrations is in a

geometric or logarithmic series, in this case with each successive concentration being 50% of the previous one, and a high concentration of 45%, the highest that can be obtained by means of the basic technique. The description which follows uses a standard *Microbics* procedure with four concentrations.

Other series of concentrations might be more suitable for some kinds of samples, and are encouraged despite the standard instructions given below for four concentrations. As the simplest variation, lower concentrations could easily be added in the same series of 50% dilutions, by continuing exactly the same dilution procedures using extra cuvettes in the wells in row C. For samples of low to moderate toxicity, a more closely-spaced series of six concentrations from about 18% to 45% has been found useful for routine testing in some laboratories.¹⁸

Test solutions are readied by the following steps.

- (a) Add 1000 μ L (1 mL) of *Reconstitution Solution* to the cuvette in the *Precooling Well*, using a micropipettor. (This

prepares for a subsequent reconstitution of a vial of *Bacterial Reagent* (Subsection 4.3.1) by allowing the *Recon* to come to temperature during the following steps.)

- (b) Add *Diluent*. Pipette 1000 μ L of *Diluent* into each cuvette in wells A1 (to become the control), A2, A3, and A4 (to become dilutions of the sample). Pipette 500 μ L of *Diluent* to each of the five cuvettes in Row B.
- (c) Prepare the (eventual¹⁹) 45% sample in cuvette A5 by adding 200 μ L of *MOAS*, then 2000 μ L (2.0 mL) of sample. Mix five times by aspirating liquid back into the 500 μ L micropipettor used to add the sample, and then dispensing it again into the cuvette. Discard 200 μ L.
- (d) Prepare the dilutions in cuvettes A2 to A4. Transfer 1000 μ L from cuvette A5 to cuvette A4, and mix with pipettor. Transfer 1000 μ L from A4 to A3, then from A3 to A2 in the same way. After the mixing of A2, discard 1000 μ L to bring it to the same volume (1.0 mL) as A1, and A3 to A5, to achieve parallel treatment of the concentrations including

18 The suggestion is from Mr. R. Salahub of Edmonton, Alberta. For example, an approximate logarithmic series of concentrations of 18, 22.5, 27, 31.5, 38.25, and 45% might be obtained by (a) bringing an aliquot of sample to proper salinity with *MOAS*, (b) adding to the cuvettes of row A, that adjusted sample in volumes 0.80, 1.0, 1.2, 1.4, 1.7, and 2.0 mL, (c) making up volumes in those cuvettes to 2.0 mL with *Diluent*, and (d) continuing with the regular procedure of transfer to cuvettes in row B. Note that older versions of the computer program for the Model 500 Analyzer were limited to two-fold dilutions, but that restriction does not exist for recent software, Version 6.0 (Section 5.2).

19 Statements can be found in the literature or in older manuals that the top concentration is 45.45%, but that is not correct for the standard procedure. The sample is originally 90.91% in cuvette A5. That is diluted half and half in cuvette A4, achieving 45.45% sample, but that is not used for testing. Using the highest concentration as an example of calculations, 0.5 mL of 90.91% sample from cuvette A5 is added to 0.5 mL of *Diluent* and 0.01 mL of *Reagent* in cuvette B5. Upon that addition, the toxicity test begins, and the concentration in cuvette B5 is:

$$90.91\% \times 0.5 / (0.5 + 0.5 + 0.01) = 45.004\%.$$

Successively lower concentrations are dilutions of this by a factor of two, and those are the concentrations used in this report.

temperature equilibrium in the well. Allow another five minutes for the contents of the cuvettes to come to the correct temperature.

4.3 Beginning the Test

4.3.1 Reconstituting Bacteria²⁰

(a) Obtain a vial of *Bacterial Reagent* from the freezer. Reconstitute it immediately by thoroughly mixing the lyophilized bacteria with the *Recon* from the *Precooling Well*, then returning the cuvette to that well. This must be done by quickly inverting the cuvette of *Recon* and rapidly pouring the *Recon* into the vial of *Reagent*. (Slow addition can result in lysing of the bacteria.) Quickly swirl the vial three or four times, then pour the mixture back into the cuvette that had contained the *Recon* and place it in the cooling well. The contents should be further mixed by drawing them into a clean 500 μ L pipette and expelling them back, 20 times. The *Reconstituted*

Reagent now in the *Precooling Well* is theoretically enough for 20 standard tests. Light emission should remain strong for two hours. If the *Reagent* is to be used for longer than that, it is advisable to include a reference toxicant in the test, and to watch for other indications of a problem of reduced light emission.²¹

- (b) Set up diluted *Bacterial Reagent* in the cuvettes of Row B. Add 10 μ L of the *Reconstituted Reagent* (about 1 million bacteria) to each of the five cuvettes in Row B, which already contain *Diluent*. (Addition of the bacteria must be made below the surface of the *Diluent* as described in the major Microtox manuals.)
- (c) Wait 15 minutes for the light production to reach a relatively steady state (slowly decreasing).

20 It is usually a more efficient use of time to reconstitute the bacteria near the start of preparations, and, while waiting 15 minutes (step 4.3.1 c), prepare the test solutions as indicated in Subsection 4.2.2.

21 The *Reconstituted Bacteria* continue to emit light for some hours. The maximum length of time for use of the bacteria will vary. Beckman (1982) states that "the light level and stability of the Microtox *Reagent* will remain satisfactory for several hours after reconstitution." He also states that mixing bacteria into cuvettes (Subsection 4.3.1, step (b)) "should begin within five minutes of reconstitution", because sensitivity to some toxicants might change with time after reconstitution. Recent documents state that the "sensitivity of the reagent is essentially unchanged for 2 hours after reconstitution" (Microbics, 1989b), or "for 1 to 2 hours after reconstitution", with sensitivity monitored in any longer use, by conducting a standard phenol test (Microbics, 1991b). Qureshi *et al.* (1983) state that tests should begin within one hour of reconstitution; they found that sensitivity increased after that, as light emission declined.

Using the Model 2055 *Toxicity Analyzer*, viability of newly reconstituted *Reagent* can be judged by light output ten minutes after reconstitution; light output should read 80 or more with sensitivity set at 1X and *SPAN* turned up full (Microbics, 1988a). Manuals do not indicate whether the same criterion can be used to judge when *Reconstituted Bacteria* are past use.

Microtox users have indicated that *Reagent* can be used for up to 4 to 6 hours provided that suitable (time-related) checks are made on performance and results. Some users test samples in duplicate, or include a reference toxicant with each test, or both. Investigators using the Model 2055 *Toxicity Analyzer* should also watch for changes in span setting at start of test, amount of span adjustment needed between tests, and blank ratios. Use of these precautions is recommended, especially the tests with reference toxicant, if *Reconstituted Bacteria* are used after two hours.

4.3.2 Time-Zero Readings

The time-zero levels of light from the cuvettes of Row B must be measured before the sample is added. Then the sample and bacteria are mixed, and the toxicity test starts.

- (a) Close the turret and turn on the chart recorder. Move cuvette B1 to the *Turret Well* and read the light production, adjusting the sensitivity of the *Toxicity Analyzer* so that a reading between 80 and 90 is obtained.
- (b) Make a time-zero mark on the chart by pressing the *CAL CHECK* button of the *Toxicity Analyzer*. Without delay, read the time-zero light levels from cuvettes B1 to B5 by moving them into the *Turret Well* in turn. These observations are called by *Microbics* "I₀", signifying "light production at time zero". The values should be in the range 80 to 100.
- (c) Immediately mix 500 μ L from each cuvette of Row A to the corresponding cuvette of Row B, i.e., from A1 to B1, A2 to B2, ... A5 to B5. (This is the formal beginning of the toxicity test. The cuvettes are allowed to sit until their light levels are checked at 5 min, 15 min, etc.) Follow the order of increasing concentration indicated above, so that the same tip can be used for the micropipettor. The actual sample concentrations in the cuvettes of Row B are now as given below, and should be recorded on the report sheet.

B1	=	0.0	% (Control or Blank)
B2	=	5.63	%
B3	=	11.3	%
B4	=	22.5	%
B5	=	45.0	%

4.4 Test Observations and Measurements

The reading of light emission after exposure should be taken for each of the cuvettes B1 to B5 at 5.0 min after the fluid from the corresponding A tube was added. Read the B cuvettes in the same manner as was done for the time-zero readings, and in the same order as was used for mixing A and B cuvettes (Subsection 4.3.2). Care should be taken that each cuvette is read at exactly **five minutes** after it received the fluid from cuvette A. There must not be any sequential error in exposure times caused by faster or slower operations at 5 min than at the time of mixing. The amounts of light being emitted at five minutes are called the "I₅" readings by *Microbics*.

Repeat the readings after fifteen minutes of total exposure.

Slow-acting toxicants such as bivalent metals might be acting in samples of unknown toxicity. Another reading at 30 min is frequently desirable, and one or more additional reading(s) at 45 and/or 60 min might sometimes be useful. By following the series of readings, the investigator can determine whether (a) the light emission is still decreasing appreciably (more than a 10% change since the previous reading), in which case exposure should be continued for another reading, or (b) the decline has levelled out, in which case the test can be ended. The objective is to allow enough time for the sample to show its maximal inhibition, but not to proceed any further than necessary into the "plateau", beyond which the sample is not causing appreciable additional inhibition. Since the readings of the control (and the test concentrations) will gradually decline with time, that must be taken into consideration when evaluating the declines in the sample

cuvettes, and the best way is to use the "Gamma" statistic described in Section 4.5. In any case, it is not desirable to use an unnecessarily long exposure, and *the 15-min IC50 should be regarded as the usual standard*. In unusual cases, bacteria might recover and light production could increase at a later time; in those cases, the earlier data should be used.

If the sample is more toxic or less toxic than expected, all the concentrations might diminish the light production by more than 50%, or by less than 50%. In either case, the test should be repeated with a more suitable range of concentrations.

4.5 Test Endpoints and Calculations

The readings for light emission are to be recorded. If a chart recorder is used for this, the values may be read from the chart at a later time. One suitable format for recording data is shown in Appendix C, and a list of items to report is given in Section 10.

The endpoint is the concentration causing 50% inhibition of light, i.e., the IC50. The calculations to estimate the 5-min IC50 are those given in the following steps. The 15-min and 30-min calculations would follow the same steps.

- (a) Calculate the "Blank Ratio" which is the proportion of the initial light which remains at a given time, in the blank. This is measured in, and calculated for, the control or blank cuvette only, i.e., cuvette B1.

There is a slight natural decline in light production during the test. This must be allowed for when evaluating the loss of light caused by toxicity. The Blank Ratio provides a means for making this allowance or correction.

$$\text{Blank Ratio } R_5 = I_5 / I_0$$

where: I_0 = light emitted by the blank at time zero

I_5 = light emitted by the blank at 5 minutes

- (b) Calculate "Gamma". Gamma is the measure of light loss used in calculating the IC50. It is calculated individually for each cuvette which contains some of the sample being tested.

Specifically, Gamma for a given cuvette at a given exposure-time, is the ratio between the amount of light lost to toxicity and the remaining light at that time.²²

$$\begin{aligned} \text{Gamma} &= (\text{light lost}) / (\text{light remaining}) \\ &= [(R_5 \cdot I_0) - I_5] / I_5 = [(R_5 \cdot I_0) / I_5] - 1 \end{aligned}$$

where: I_0 = light emitted at time zero

I_5 = light emitted at 5 minutes

R_5 = Blank Ratio

When Gamma equals unity, half of the light production has been lost to toxicity, and that is the IC50.

4.5.1 Graphic Estimate of IC50

Values of Gamma are plotted on the vertical scale of logarithmic paper, against the corresponding sample concentrations on the horizontal logarithmic axis (Microbics, 1992). A line is fitted by eye, then the IC50

22 Gamma is the ratio of light lost to the amount of light remaining. A simpler measure might be used, such as the absolute amount of light lost because of toxicity, but a better linear relation is generally obtained by plotting the logarithm of Gamma against the logarithm of concentration. Gamma is therefore preferred as a method of describing the dose-effect curve and estimating IC50 and confidence limits. An example comparing the two approaches is given by Beckman (1982).

is read off as the concentration that corresponds to a Gamma of 1.0 (Figure 3).

The IC50 should be interpolated, that is, there should be at least one observation of a concentration causing more than 50% reduction of light (concentration(s) higher than the IC50), and at least one observation of a concentration causing less than 50% reduction of light. Subsection 4.8.5 provides further discussion of the need for interpolation, and of endpoints such as IC20 instead of IC50.

Accuracy of this method depends on the skill of the investigator in fitting a line by eye. For this reason, it is usually desirable to use a computer- or calculator-based method as the primary means of estimating the IC50 (see Subsection 4.5.2). Nevertheless, a graphic analysis should be considered a routine part of each test, as a check on the reasonableness of the calculated IC50. A plot of the data is particularly useful for detecting anomalies in the test results, which might suggest further investigation or might reduce confidence in the results. Any major disparity between calculated and graphically-derived IC50s should be resolved.

Hand plotting is also useful for detecting incorrect entries, if observations are being manually entered into a computer. Any error might not otherwise be detected. The *Microbics* software program will provide readouts of intermediate data and a useful plot of the results, on demand. If manually-entered results are used for a computer-generated graph, the plotted points should be checked against the observed readings to guard against errors in entry and anomalous estimates of IC50. Such error might be detected by the hand-plotted check.

4.5.2 IC50 Estimates Using a Calculator or Computer

An investigator could fit a straight line to the relation of log Gamma to log concentration by standard mathematical methods, although such steps are not covered in detail in Microtox manuals. Least-square regressions are apparently used in the calculation by the *Microbics* computer program, so any statistics textbook would provide the methods for hand calculations. Gamma and the test concentrations should first be transformed to either natural or base-10 logarithms. Note that the widely available computer programs for calculating median lethal concentrations (LC₅₀) or effective concentrations (EC₅₀) are for quantal data and are *not suitable* for the Microtox data.

Computer software from *Microbics* is recommended as the preferred method of analysis, in view of the wide availability of personal computers in Canadian laboratories. *Microbics* supplies software in BASIC that is compatible with IBM personal computers. Version 6.0 (late 1991) of this software is recommended as a replacement for any previous program for the Microtox test. It is designed for the newer Model 500 Microtox Analyzer (see Section 5), but can also be used with the Model 2055. The software automatically transfers light readings from the test to a computer, with the Model 500 Analyzer, and also with the Model 2055 if it is fitted with an adapter for that purpose.

The computer program handles a variety of procedures including various concentration ranges and, in fact, all the types of tests covered herein. There are options for printing various intermediate steps in calculations, but usually the printed output includes details of the material tested, a description of the basic test variables, concentrations and Gamma values, estimate of IC50 and confidence limits, and a plot

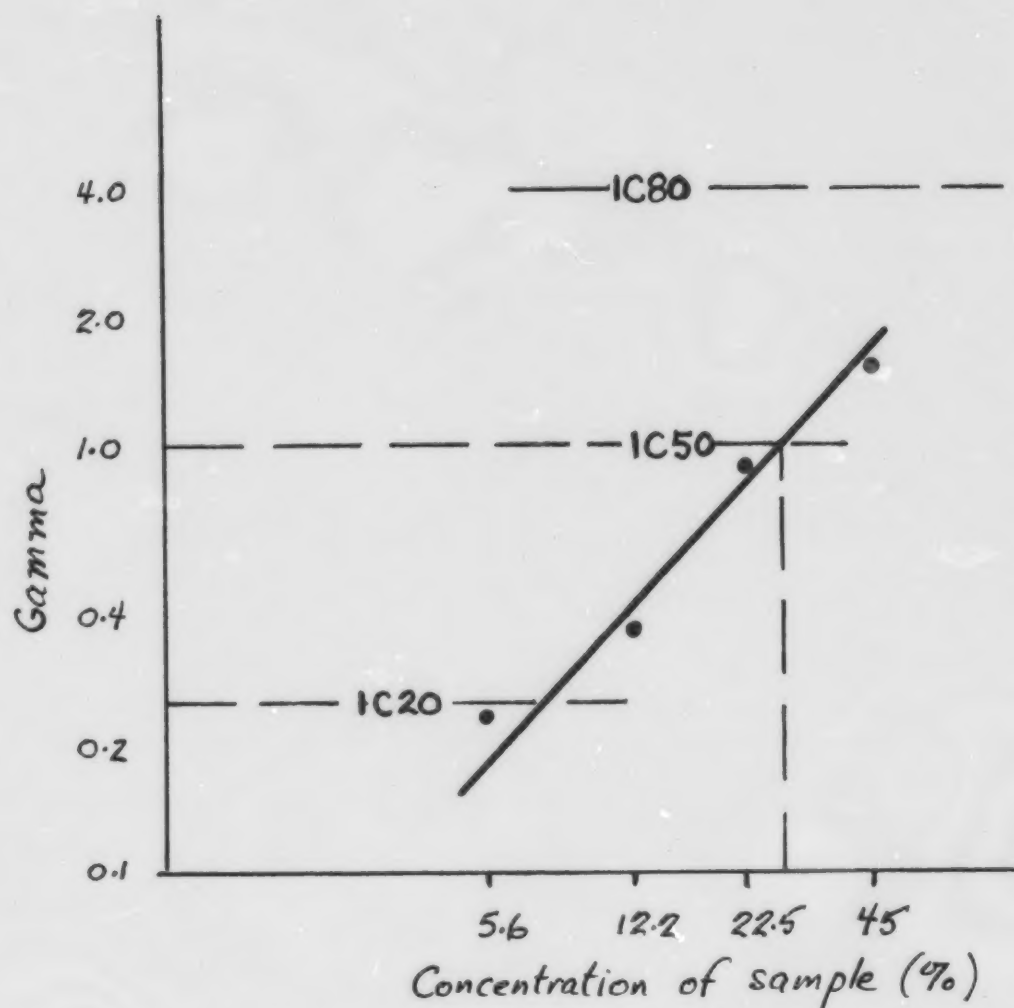


Figure 3 Example of Estimating the IC₅₀ with a Hand-drawn Graph on Logarithmic Paper

The concentration causing 50% inhibition of light production by *Photobacterium phosphoreum* can be read from the graph. In this hypothetical example there were four concentrations. The concentration expected to cause 50% inhibition can be read by following across from a Gamma value of 1.0 (broken line in graph) which represents 50% decrease, to the intersection with fitted line, then down to the horizontal axis, for an estimated IC₅₀ of about 28%. The IC₂₀ could be estimated in a similar way if desired, but higher concentrations should be tested before estimating the IC₈₀ (see Subsection 4.8.5).

indicating the observations and fitted line. When using the computer program, it is imperative to check for reasonableness of the measurements of light reduction and estimate of IC50. Printing of intermediate steps in calculations will assist in such a check.²³

The desirability of basing calculations on measured values higher and lower than 50% light reduction is discussed in Subsection 4.8.5. Other endpoints which might be useful, such as IC20 are also discussed in this subsection.

4.6 Reference Toxicant

The routine use of a reference toxicant or toxicants is necessary to assess, under standardized test conditions, the relative sensitivity of the bacteria after reconstitution, the accuracy of dilution techniques, and other factors affecting the precision and reliability of data produced by the laboratory for the reference toxicant(s) (Environment Canada, 1990d). Reference toxicant(s) should be tested at least once a month during periods when Microtox tests are being run, and initially upon first use of a new shipment or batch of *Bacterial Reagent*. Test conditions and procedures should be the routine ones outlined herein and in *Microbics* literature.

Criteria considered in recommending appropriate reference toxicants for this test include:

- a chemical, readily available in pure form;

- stable (long) shelf life;
- highly soluble in water;
- stable in aqueous solution;
- minimal hazard posed to user;
- easily analyzed with precision;
- good dose-response curve for Microtox; and
- any influence of pH on toxicity, if it is known.

Four chemicals are recommended as suitable reference toxicants for the Microtox test and one or more of them may be used within a laboratory.

Reagent-grade phenol is one suitable reference toxicant. For phenol, *Microbics* advises that a 5-min IC50 in the range 13 to 26 mg/L should be expected at 15°C. Kaiser and Ribo (1988) tabulate eleven results from the literature showing a mean 5-min IC50 of 30 mg/L. A stock solution of phenol should be made up on the day of use.

Zinc sulphate or potassium dichromate may also be used as the reference toxicant, and both have been found satisfactory in Canadian laboratories. Zinc is recommended by *Microbics*, with an estimated 5-min IC50 in the range 1.4 to 2.7 mg Zn/L at 15°C. The chromium in potassium dichromate is carcinogenic and should be handled with suitable precautions (Environment Canada, 1990d). Sodium

23 The Western Canada Microtox Users Committee has analyzed data using different computer programs and found that they yield very similar results. For extrapolated data, however, different programs show more variability in results. It is appropriate for investigators to carry out within-laboratory checks by different methods, and to compare techniques and results with other laboratories, through informal groups such as the Users Committee.

lauryl sulphate is another possible choice. It is recommended by BNQ (1987) with suggested test concentrations spanning the range 0.5 to 25 mg/L; Kaiser and Ribo (1988) list seven IC50s with a geometric mean value of 1.3 mg/L. This substance is not included in the list of reference toxicants recommended by Environment Canada (1990d).

Concentration of reference toxicant in a stock solution should be measured by appropriate chemical methods (e.g., APHA *et al.*, 1989). Calculation of IC50 should be based on the measured concentration if it is appreciably different (i.e., $\geq 20\%$) from the nominal one, and if the accuracy of the chemical analyses is satisfactory.

A warning chart (Environment Canada, 1990d) should be prepared and updated for each reference toxicant used. Successive IC50s should be plotted on this chart, and examined to determine whether the results are within ± 2 SD of IC50s obtained in previous tests. The geometric mean IC50 with its upper and lower warning limits (± 2 SD calculated on a geometric [logarithmic] basis)²⁴ are recalculated with each successive IC50 until the statistics stabilize (U.S. EPA, 1989a; Environment Canada, 1990d).

If a particular IC50 falls outside the warning limits, the reagents, test system, or technique are suspect. Since this might occur 5% of the time due to chance alone, an outlying IC50 does not necessarily mean that the sensitivity of the *Reagent* or the precision of toxicity data produced by the laboratory are in question. Rather, it provides a warning that this might be the case. It would be wise to carry out a thorough check of all test

conditions, including use of a new supply of *Bacterial Reagent*.

Use of warning limits does not necessarily indicate that a laboratory is generating consistent results. A laboratory that produced extremely variable data for a reference toxicant would have wide warning limits; a new data point could be within the warning limits but still represent undesirable variation in results obtained in tests. A coefficient of variation of 20% or 30% is tentatively suggested as a limit by Environment Canada (1990d). Although that seems a reasonable range, establishing firm limits for allowable variation would require evaluation of the reproducibility that Canadian laboratories can achieve in testing various reference toxicants with Microtox, under normal operating conditions.

4.7 Legal Considerations

Bacterial toxicity tests have been specified for an industry in at least two Canadian provinces, at the date of compiling this report. Considerations on regulatory use of Microtox are therefore in order. Specification of endpoints of the test, for legal purposes, is beyond the scope of this report; however, certain useful generic practices are addressed.

If test data are likely to be used for prosecution, care must be taken to ensure that samples are collected and tested in ways that are admissible in court. For this purpose, legal samples must be: representative of the substance being sampled; uncontaminated by foreign substances; identifiable as to date, time, and location of origin; clearly documented as to the chain of custody; and analyzed as soon

24 If the IC50s fail to show a lognormal distribution, an arithmetic mean and SD might prove more suitable.

as possible after collection. Persons responsible for conducting the test and reporting the findings must maintain continuity of evidence for court proceedings (McCaffrey, 1979), and ensure the integrity of the test results.

4.8 Variations of Basic Procedure

4.8.1 Range-Finding Tests

With a sample of unknown toxicity, it will usually be helpful to obtain an approximate estimate of toxic strength in a preliminary test. Results might show that the final test should be run with concentrations that are lower than the standard ones, or in a narrower range (smaller ratio between the concentrations, see Subsection 4.2.2).

Such a preliminary range-finding test (Microbics, 1983 No. M101; 1988a) can be done quickly by adding 10 μL of sample to a standard cuvette which contains the usual 500 μL of *Diluent* and 10 μL of *Reconstituted Reagent*, producing a sample concentration of about 2%. A 60% reduction of light in five minutes would indicate an extremely toxic sample. A ten-to-one dilution of the original sample could be used in a final test.

In general, a light reduction of 60% in five minutes in the preliminary tests, would suggest that a ten-to-one dilution is necessary before the standard Microtox preparations (Section 4.2) are carried out. A light reduction >20% and <60% in the range-finder would suggest an initial 5-to-1 dilution. The range-finding test is fast enough that it could be done during the waiting period for light stabilization, using some extra cuvettes of *Diluent* and *Reconstituted Reagent*, made up in Row C of the *Toxicity Analyzer*.

4.8.2 Replicates

A replicate of the control is recommended as a useful safeguard, since an anomalous reading from a single control cuvette would bias the overall result.

If desired, replicates of the test concentrations may be run, although general experience in Canadian laboratories does not suggest that this is necessary (see Subsection 3.1.1). Replicates could be run in the wells of Row C, using the same techniques as used for the cuvettes in Row B. In such a case, there should be increases in the amounts of liquid added to cuvettes of Row A. In Subsection 4.2.2, the following changes would be made to the standard procedure. In step (b), 1500 μL of *Diluent* would be added to cuvettes A1 to A4, instead of 1000 μL . In step (c), cuvette A5 would receive 3000 μL of sample and 300 μL of *Microtox Osmotic Adjustment Solution (MOAS)*. Transfers in step (d) would be 1500 μL instead of 1000 μL . Subsequently in Subsection 4.3.2 (c), transfers to Row C would be made from Row A in the same way and with the same volumes of fluid, as was specified for transfers to Row B.

4.8.3 Full-Strength Sample

The highest concentration in the standard procedure is 45% (Section 4.2), but sometimes an investigator would wish to test a sample at full strength or nearly so. It is possible to test 90% sample by omitting *Diluent* and adjusting salinity of the sample with *MOAS*. Alternatively, a sample can be tested at almost full strength by omitting both *Diluent* and *MOAS*, and using analytical grade, crystalline sodium chloride to bring the sample to 2% salinity. Light production at time zero cannot be measured in this case, since the *Reagent* is added directly to the sample. The decrease in light

production in each dilution of sample is estimated by comparing with a blank of *Diluent*.

Microbics (1989b) suggests that testing at full strength should not be used for samples having appreciable toxicity (i.e., toxicity that is still measurable in dilutions); there could be loss of precision because the full-strength tests are very sensitive to the technique used by the operator.

The procedure for testing a sample at full strength is actually very similar to that given in steps (a) to (d) in Subsection 4.2.2, and steps (a) to (c) in Subsection 4.3.1, but is repeated below, essentially as given by Microbics (1989b). One difference is that only one set of cuvettes (A1 to A5) is used in the following method. A similar method for several replicates of 99% sample is outlined by Dutka (1988).

- (a) Add 1000 μL *Recon* to a cuvette in the *Precooling Well*.
- (b) Add 1000 μL of *Diluent* to A1, A2, A3, and A4.
- (c) Add 200 μL *MOAS* to A5, for the 90% sample.
- (d) Add 2000 μL of sample to A5, mix, then discard 200 μL .
- (e) Transfer 1000 μL from A5 to A4, and mix A4.
- (f) Transfer 1000 μL from A4 to A3, and mix A3.

- (g) Transfer 1000 μL from A3 to A2, mix A2 then discard 1000 μL .
- (h) Wait five minutes for temperature equilibration.
- (i) Reconstitute a vial of bacteria in the usual way (Subsection 4.3.1 (a)).
- (j) Start a timer. Transfer 10 μL of the *Reconstituted Reagent* to each of the five cuvettes A1 to A5²⁴, and mix in the same order with a 500 μL pipettor.
- (k) After 5 minutes and 15 minutes, read the light emissions from each cuvette, and make tabulations and calculations by comparing A2 to A5 with the blank A1. The concentrations from high to low are 90.0%, 45.0%, 22.5%, 11.3%, and 0%.

To test a "full-strength" sample, do not add *MOAS* to A5 in step (c). Instead, add 40 mg of dry sodium chloride to A5, and dissolve it in the added sample. Do not discard fluid from A5 in step (d). The test concentration in cuvette A5 is close to full strength. Allowing for the addition of *Reagent*, the sample concentration can be considered to be 99%.²⁶

4.8.4 Sucrose as the Osmotic Adjusting Agent

The sensitivity of the Microtox system to certain metals and ammonia can be increased by using sucrose instead of sodium chloride, as the base of the *Diluent*. Microbics indicates (Microbics, 1988c) that the IC50 decreases by a factor of 34 for a salt of cadmium, a factor of 36 for a salt of

25 It is important that the stock of *Reconstituted Reagent* should not be contaminated by toxic sample carried back by the pipette. The tip must be changed after each transfer.

26 If calculated on the basis of weight, with allowance for the salt added, the concentration tested would be 97.1%.

ammonia, 15, 10, and 10-fold for salts of nickel, copper, and zinc, but with little change for salts of arsenic, chromium, and mercury. At the same time, the *Sucrose Diluent* did not adversely affect sensitivity of Microtox to other kinds of toxicants tested (phenol, octanol, xylene and potassium cyanide). Tests by Ankley *et al.* (1990) confirmed major increases in sensitivity to zinc and nickel when using sucrose, but little difference for copper, and similar results for most of 44 whole effluents.

This adaptation of the Microtox procedure would obviously be suitable for testing metals and ammonia as pure chemicals. It could also be useful as an addition to the standard procedure, for effluents and other environmental samples suspected of having certain metals or ammonia as major contributors of toxicity.

The procedure involves little change from the standard one. Instead of *MOAS*, 2 g of solid sucrose (ACS grade) is added to 10 mL of sample; 2 mL of that modified sample is put into cuvette A5. Instead of the usual *Diluent*, 1000 μ L of *Sucrose Diluent* (20% sucrose) is added to each of cuvettes A1 to A4, and 500 μ L to each of cuvettes B1 to B5. Serial dilution, mixing with *Reagent*, and reading of results then proceeds as with the standard test. The percent concentrations tested in cuvettes B2 to B5 are respectively 6.19, 12.4, 24.8, and 49.5.

4.8.5 Alternate Endpoints

With any method of estimating the IC₅₀, whether graphic or fitting a line by calculator

or computer, it is possible to estimate other percent effects, such as IC₂₀ or IC₈₀. Although IC₅₀ is recommended here as a standard measure of effect, estimates of other percent effects might be appropriate for particular purposes and could be used. The IC₅₀ should always be included in the report if it can be estimated. The 95% confidence limits should also be calculated for any estimates of other percent effects. The procedure should not be taken to extremes; it would be illusionary to attempt to calculate some sort of "threshold of toxic effect" as the IC₁ or IC_{0.1}, etc. The conclusion of Quebec Environment from a large set of tests, reviewed in Subsection 3.1.1, was that a 17% inhibition of light was the minimum that could be quantified with statistical significance (BNQ, 1987). A 20% effect corresponds to a Gamma of 0.25, an IC₈₀ corresponds to a Gamma of 4.0, and other values are given in Beckman (1982).

Estimates of a particular "inhibiting concentration" (IC₅₀, IC₈₀, etc.) should properly be made only when percent effects were obtained in the test at higher and lower concentrations. For any toxicity test, the most reliable endpoint of effect will be within the range of concentrations that was tested and provided useful information. In particular, it is not desirable to extrapolate in order to estimate an IC₅₀ from data that show only less than 50% effect. Accordingly, it is recommended here, that to be considered valid for purposes of reporting to Environment Canada, an IC_p should have at least one data point that is $>p$.²⁷

27 *Microbics* has argued (1983, No. M110) that extrapolation can be done with relative safety for the Microtox test because it measures a rate of biological activity rather than a quantal effect such as mortality of fish in a lethal test. There is some truth in the assertion, but it might not always be correct to assume that a linear relation in low concentrations will extend with unchanged slope into high concentrations, or vice versa. For example, Beckman (1982) shows some non-linear logarithmic plots of Gamma versus concentration. Canadian investigators report some such irregular curves.

Some information can be gleaned from extrapolations, particularly since the highest valid IC50 would be about 45%, in the standard procedure which has 45% as the highest concentration tested. Obviously it could be said that "the IC50 was greater than 45%". Alternatively, it might be possible and useful to estimate the IC20 rather than the IC50. An ICp obtained by extrapolation might be used for general comparative purposes, such as the relative degree of toxicity of different effluents. Any such values should be clearly labelled as "predicted IC50", "extrapolated IC50", or other suitable qualifier.

4.9 Procedure for Coloured or Turbid Samples

Various concentrations of sample should be tested for toxicity in the usual way, then corrected by the following method. The procedure uses a double cuvette, i.e., a narrow tube fixed within a normal one, the *Colour Correction Cuvette (CCC)*. It is obtainable from *Microbics* and is reusable. An aspirator that comes with the tube, or other long-tipped pipette, is used to manipulate the liquids without generation of air bubbles. This procedure is not suitable for light-reflecting samples, nor for the *Model 500 Analyzer* which requires different volumes.

After the regular test with the sample, the following steps are carried out.

- (a) Add 1500 μL of *Diluent* into the outer chamber of the CCC and install it in C1. Add 1000 μL of *Diluent* to a standard cuvette in well C2, and 1500 μL to C4.
- (b) Add 1500 μL of a selected concentration of sample into a standard cuvette in well C3. Normally, the concentration would be the highest one that yielded usable results in the test just completed, or the concentration closest to the IC50 just determined. Wait at least five minutes for thermal equilibrium.
- (c) Add 50 μL of *Reconstituted Reagent* to C2 and mix. Wait at least 10 minutes for stabilization of light emission.
- (d) Using aspirator, add liquid from C2 to inner chamber of CCC until it equals the height of liquid in the outer chamber.
- (e) Move CCC to the *Turret Well*. Adjust photocell to read about 90% and record until a stable rate of decrease is evident, then use the last four minutes for calculations.
- (f) Without moving the CCC, remove *Diluent* from the outer chamber with the aspirator, then pipet 1500 μL of the test concentration from C3 to the outer chamber. Record light emission until a stable rate of decrease is evident, again using the last four minutes for calculations.
- (g) Without moving the CCC, remove sample from the outer chamber with the aspirator, then refill with *Diluent* from C4. Record light level for at least three minutes.
- (h) On the chart record, measure loss of light transmission caused by the sample, by using the graphic method in *Microbics* (1988b). The reading with the sample is called I_r , and is simply the top right (final) value of the reading recorded in

step (f). The light transmission with *Diluent* is called I_p , and is interpolated to the time of reading the sample by drawing straight lines on the three readings which were traced on the chart in (e), (f) and (g). One line is drawn to join the top right-hand (final) ends of readings (e) and (g), and another straight line is drawn vertically up from the top right of reading (f). The intersection of those lines yields I_p , the light transmission with *Diluent*.

If there are several samples to be run through the colour-correction procedure, they may all be done sequentially before taking the second reading of *Diluent* in step (g). In that step, after removing the sample from the outer chamber, replace it with the next sample, take a reading as before, and continue through all the samples. Finally, complete step (g) by reading with *Diluent* in the outer chamber. The graphic estimates in step (h) will use a series of vertical lines drawn up from the top right of sample readings, to determine a series of *Diluent* interpolations (I_p) to be used in corrections of the respective samples.

The correction of the IC50 involves constants derived from the geometric proportions of the CCC. *Microbics* recommends using calculator or computer programs to do the arithmetic (Subsection 4.5.2). The following formulae for doing the calculations by hand are given by BNQ (1987), and by Microbics (1988a) in a slightly modified format.

Calculate A_c :

$$A_c = 3.1 \ln (I_p/I_r)$$

For any concentration C_x used in a test, the absorbance due to that concentration of sample is called A_x and should be calculated as:

$$A_x = A_c (C_x/C)$$

Convert that absorbance into the transmittance expected at the test concentration T_x , by calculating:

$$T_x = (1 - e^{-A_x})/A_x$$

Calculate a corrected value for Gamma, G_c , from the Gamma originally observed for the test concentration, G_x , as:

$$G_c = T_x (1 + G_x) - 1$$

where: I_0 = light production at time zero
 I_5 = light production at five minutes
 I_r = light production by the sample (C) used in a colour-correction test (obtained from the chart)
 I_p = light production for the diluent in a colour-correction test (obtained by interpolation on the chart)
 C = concentration of sample used for the colour correction procedure
 A = absorbance
 A_c = absorbance from the correction at concentration C
 C_x = any (specified) concentration used in a test
 A_x = absorbance at concentration C_x
 T_x = calculated transmittance at concentration C_x
 e = base of natural logarithms
 G = Gamma
 G_x = the value of Gamma originally observed for the test concentration C_x
 G_c = the corrected value of Gamma

The corrected Gammas for the test concentrations are then used to calculate a

corrected IC50. This assumes linearity of colour interference at all concentrations, which occasionally might not be true. Absorbance could be measured at each

concentration, but that is seldom warranted; at most, a second check might be run at a high concentration of sample, as discussed by Beckman (1982).

Section 5

Procedures for Model 500 Analyzer

All the procedures in Section 4 apply to the Microtox Model 2055 *Analyzer*, which has been the most common model used until recently in Canadian laboratories. Many of the concepts and general methods described in Section 4 also apply to the newer Model 500 *Analyzer* (1989), but some differences exist. These are listed in the following text and are shown in Figure 4 and Table 2.

5.1 General Changes

The major differences in the Model 500 *Analyzer* (Table 2) are:

- (a) The Model 500 automates most of the functions in the *Analyzer*, including lowering a given cuvette into position for reading light emission, at the touch of a button. This helps to standardize the timing and pace of readings. However, the new model could also be operated manually, if desired, for any procedure that is used on the Model 2055.
- (b) The Model 500 accommodates 30 cuvettes instead of 15. This allows three samples to be tested simultaneously if desired.
- (c) The new *Analyzer* is designed to send data to a computer and/or a chart recorder. The computer software available from *Microbics* in late 1991 (Version 6.0) is flexible, with many options for running different series of concentrations, duplicates, manual or automatic input of data, etc. This software can also be used on the Model

2055 *Analyzer* if it has the computer interface.

The *Analyzer* is similar to the Model 2055 (Figure 2), but the Model 500 has wells for 30 cuvettes, has no projecting turret, and lacks adjustment knobs. A computer would likely receive data from the test, rather than a chart recorder.

5.2 Specific Procedural Changes for Model 500 Analyzer

Procedures are detailed in a recent manual from *Microbics* (1992). In general, the kinds and volumes of fluids manipulated, and the timing, remain the same as given in the preceding sections. Some steps of the manual procedure are no longer required, and those items are given below, with the initial codes referring to preceding sections of this document. Some general annotations and recommendations are given with the descriptions of changes.

3.2.2 (d). The chart recorder will not likely be selected as an option by investigators, since it has been rendered rather obsolete for the new *Analyzer*. Model 500 will send readings to a micro-computer (Subsection 3.2.2 (e)) for recording and later processing. Alternatively, the light readings can be read from a display on the front of the *Analyzer* and recorded manually. The Model 500 adjusts readings to relative values from 0 to 199. During initial standardization, a value of zero is assigned to a reading in darkness, and a value close to 95 is assigned to the reading of the Reagent Blank.

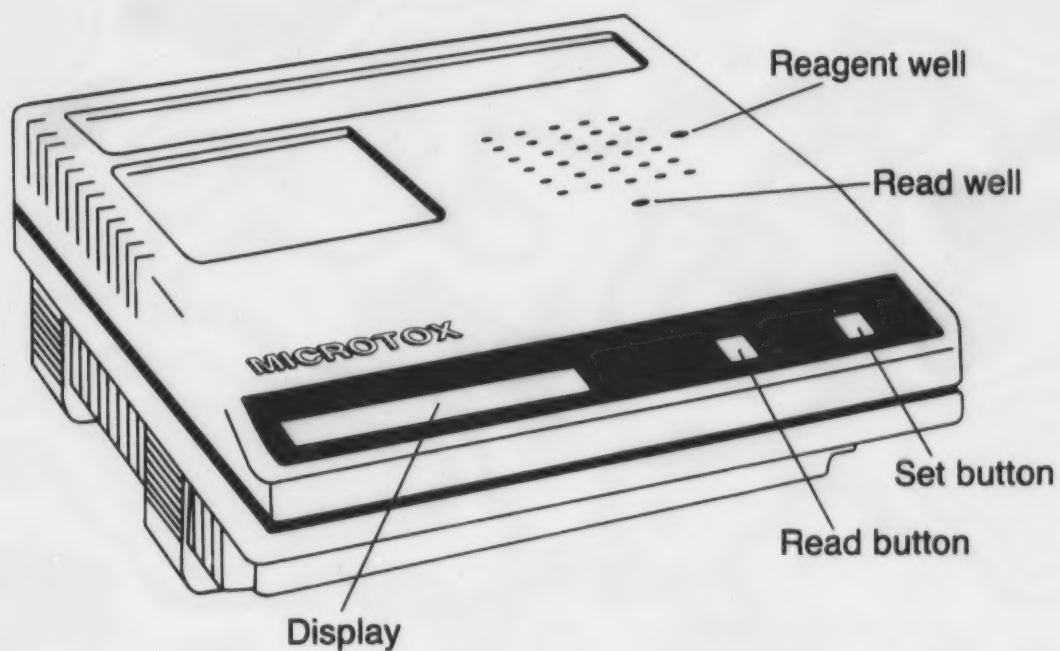


Figure 4 **General Appearance of the Model 500 Analyzer for the Microtox Test**

This *Analyzer* is similar to Model 2055 (Figure 2), but Model 500 has wells for 30 cuvettes, has no projecting turret, and lacks adjustment knobs. A computer would likely receive data from the test, rather than a chart recorder.

Table 2 Comparison of Models 2055 and 500 Microtox Analyzers

Item	Model No.	
	2055	500
Number of incubator wells	15	30
Span control	Manual	Automatic
Temperature control	Manual	Automatic
Zero adjustment	Manual	Automatic
HV (high voltage) check	Manual	Automatic
CAL check	Manual	Not needed
Cuvette feed	Manual	Automatic
Computer port	Optional	Yes
Recorder port	Yes	Yes
Turret check	Manual	Automatic
Incubator check	Manual	Automatic
Air check	Manual	Automatic
Sensitivity setting	Manual	Automatic

4.2.1 (a) to (e). The *Analyzer* checks temperatures automatically and signals the operator with a warning light if there is deviation from desired temperature. The steps for calibrating the *Analyzer* are carried out by the machine when the SET button is pressed.

4.2.2 The *Microbics* computer software Version 6.0 allows for diverse series of concentrations. Older versions of the computer program required two-fold dilutions, and users of those early versions would be well advised to obtain recent software.

4.3.2 (b). It is unlikely that timing will be marked on a chart record. If direct computer input is used, the program signals the appropriate times for light readings. Otherwise, a timer is used.

4.5 (a) and (b). Calculations of the "blank ratio" and "Gamma" will most likely be

done by a computer program (see Subsection 4.5.2). The investigator should obtain the computer-generated plot. All of these data and calculations should be inspected for reasonableness. Any apparent anomalies should be checked, even to the point of making hand calculations or hand-fitted lines. In particular, if the estimated IC_p is above the highest concentration tested, it is not considered a valid estimate for purposes of reporting to Environment Canada. Such an IC_p can, however, be labelled as "tentative", "extrapolated", or other similar qualifying term, and used for general comparative purposes (see Subsection 4.8.5).

4.9. For a colour-correction procedure, the general approach, manipulations, and readings are similar to those for the Model 2055 but differ in detail. No graphic analysis is done by measuring on a chart, but a corrected value for I₀ is calculated arithmetically and used in the programs to estimate an IC₅₀ or IC_p.

Section 6

Specific Procedures for Testing Chemicals

This section gives particular instructions for testing chemicals, in addition to the procedures listed in Sections 4 and 5.

6.1 Properties, Labelling, and Storage of Sample

Information should be obtained on properties of the chemical to be tested, including water solubility, vapour pressure, chemical stability, dissociation constants, and biodegradability. Datasheets on safety aspects of the test substance should be consulted, if available. Other available information such as structural formula, degree of purity, nature and percentage of significant impurities, amounts of additives, and n-octanol:water partition coefficient should be obtained and recorded.²⁸ An acceptable analytical method should also be known for the chemical in water at concentrations intended for the test, together with data on precision and accuracy. If the chemical's aqueous solubility is in doubt or problematic, acceptable procedures used previously for preparing aqueous solutions should be reported.

Chemical containers should be sealed and coded or labelled (chemical name, supplier, date received, person responsible for testing, etc.) upon receipt. Storage conditions (e.g., temperature, protection from light) are frequently dictated by the nature of the chemical. Standard operating procedures for chemical handling and storage must be followed.

6.2 Preparing Test Solutions

If a new pesticide or similar category of chemical were being tested under a formal program of regulations for registering the chemical, those regulations might require the use of replicates of test concentrations.

The "sample" to be tested will be a stock solution, or diluted from a stock solution that was prepared by dissolving the chemical to be tested in deionized water. The concentration of the test chemical in the solution should be measured (Section 6.3), and stability of the chemical determined, before the test. Stock solutions subject to photolysis should be shielded from light, and unstable solutions must be prepared at a suitably short time before use. The sample should be adjusted to 2% salinity [Subsection 4.2.2 (c)] at the beginning of a test. The sample should meet the requirements for colour, turbidity, pH, and dissolved oxygen content specified in Section 4.1. The Microtox assay can then proceed as in the Universal method of Section 4.

For chemicals that do not dissolve readily in water, stock solutions may be prepared using the generator column technique (Billington *et al.*, 1988; Shiu *et al.*, 1988) or, less desirably, by ultrasonic dispersion. Use of ultrasonics can produce droplets that differ in size and uniformity, some of which could migrate towards the surface of the liquid, or vary in biological availability

²⁸ Knowledge of the properties of the chemical will aid in determining any special precautions to be taken while handling and testing it (e.g., testing in a well-ventilated facility). Information on chemical solubility and stability will also be useful in interpreting test results.

creating variations in toxicity. There could also be effects on light transmission, which is of major importance in the Microtox test. Organic solvents, emulsifiers, or dispersants should not be used to increase chemical solubility except in instances where they might be formulated with the test chemical for its normal commercial purposes. If used, an additional control solution should be prepared containing the same concentration of solubilizing agent as in the most concentrated solution of the test chemical. Such agents should be used sparingly, and should not exceed 0.1 mL/L in any test solution. If solvents are used, the following are preferred: dimethyl formamide; dimethyl sulphoxide (DMSO);²⁹ triethylene glycol; methanol; ethanol; and acetone (U.S. EPA, 1985).

6.3 *Observations and Measurements on Sample*

During preparation, each stock solution should be examined for evidence of chemical presence and change (e.g., solution colour and opacity, precipitation or flocculation). Any observations should be recorded.

It is desirable to measure the concentration of the chemical in the stock solution.³⁰ Aliquots should be preserved, stored (if necessary), and analyzed chemically according to best proven methodologies available. If a measured concentration is available, it should be used to calculate and express the toxicity, unless there is good reason to believe that the measurement is not accurate.

29 Keep DMSO in its liquid form at 19°C or higher. If repeatedly frozen and thawed its toxicity might increase.

30 Such analyses need not be undertaken in all instances, due to cost, analytical limitations, or previous technical data indicating chemical stability in solution under conditions similar to those used in preparation and in the test. Chemical analyses are particularly advisable if the test material is volatile, of low solubility, precipitates, or sorbs to the container.

Section 7

Specific Procedures for Testing Samples of Effluent, Leachate, and Elutriate

Particular instructions for testing samples of effluent, leachate, and elutriate, in addition to the procedures listed in Sections 4 and 5, are given in this section. Note that some tests of sediments can involve separate tests of interstitial water following the procedures used for an elutriate.

7.1 Sample Labelling, Transport, and Storage

Containers for transportation and storage of samples of effluent, leachate, or elutriate must be made of nontoxic material. For large samples intended for tests with larger organisms as well as Microtox, glass or Teflon™-coated containers are preferred, as they are inert and reduce sorption of chemicals. Polyethylene or polypropylene containers manufactured for transporting drinking water are less desirable but may also be used. For smaller samples intended only for the Microtox test, borosilicate glass containers are recommended, with screw-caps lined with Teflon or other closure known to be nontoxic.

Samples of 500 mL to 1 L are recommended for the Microtox test, to allow portions to be used for measuring initial pH, dissolved oxygen, and other characteristics (Subsections 4.1.2, 4.1.3; and Section 7.4). All containers must either be new or thoroughly cleaned, and rinsed with uncontaminated water. They should also be rinsed with the sample to be collected. Containers should be filled completely to minimize air space.

Upon collection, each sample container must be sealed and labelled or coded. Labelling must include at least sample type, source, date and time of collection, and name of sampler(s). Unlabelled or uncoded containers arriving at the laboratory should not be tested. Nor should samples arriving in partially filled containers be routinely tested, since volatile toxicants escape into the air space. However, if it is known that volatility is not a factor, such samples might be tested at the discretion of the investigator.

Testing of effluent and leachate samples should commence as soon as possible after collection. Whenever possible, testing should begin within 24 h, and must commence no later than 72 h after sampling. Samples of sediment or soil collected for extraction and subsequent testing of the elutriate should also be tested as soon as possible; extraction procedures should begin within two weeks of sampling (preferably within one week), and testing must start no later than six weeks after collection (Environment Canada, 1993). Procedures given in Environment Canada (1993) for the preparation of elutriates should be followed. Testing of such elutriates must commence within 72 h of their preparation, or as specified in a regulation or protocol.

All samples of effluent or leachate should be kept cool (1 to 7°C, preferably $4 \pm 2^\circ\text{C}$) throughout their period of transport. Upon collection, warm ($>7^\circ\text{C}$) samples should be cooled to 1 to 7°C with ice or frozen gel packs. As necessary, gel packs or other means of refrigeration should be used to

assure that sample temperature remains within 1 to 7°C during transit. Samples must not freeze during transport. Samples should be stored in darkness in sealed containers at $4 \pm 2^\circ\text{C}$. Sub-samples for testing should be brought to 15°C immediately before the test.

Temperature conditions should also be as indicated for transportation and storage of elutriates, as well as for samples intended for aqueous extraction and subsequent testing of elutriate, unless otherwise specified.

7.2 Preparing Test Solutions

Samples in the collection containers must be agitated thoroughly just prior to pouring to ensure the re-suspension of any settleable solids. Sub-samples (i.e., a sample divided between two or more containers) must be mixed together to ensure their homogeneity. If further sample storage is required, the composited sample (or a portion of it) should be returned to the sub-sample containers and stored (Section 7.1) until used.

The pH and dissolved oxygen content of the sample should be checked with regard to the limits in Subsections 4.1.2 and 4.1.3.

Tests for monitoring and for compliance with regulatory requirements should normally include an undiluted portion of the sample (Subsection 4.8.3) and a control solution. Regulations might require replicates of the control and some or all of the test concentrations. Toxicity tests conducted for other purposes (e.g., determination of in-plant sources of toxicity,

treatment effectiveness, effects of process changes on toxicity) may, depending on the study objectives, be single- or multi-concentration tests. Single-concentration tests are often cost-effective for determining the presence or absence of toxicity or as a method for screening many samples for relative toxicity.

7.3 Dilution Water

For freshwater discharges of effluents, and freshwater leachates and elutriates, the *Diluent* described in the Universal procedure should normally be used in tests. Surface water from lakes, rivers, and streams is normally unsuitable for use as dilution water in the test, in an attempt to simulate a local situation.³¹

There are additional options for effluents being discharged to marine situations, and for elutriates which have been obtained using seawater, as in an evaluation of marine sediments. The choice among the options depends on the purpose for doing the test. If a "standardized" toxicity rating is desired, the Universal procedure should be followed, using the normal Microtox *Diluent* and the standard salinity of 2%. If it is desired to simulate the local situation, salinity of the sample and *Diluent* should be that of the receiving water. The *Photobacterium phosphoreum* used in the tests has maximal light production in the salinity range 2 to 3.7%.

Use of clean seawater in place of the Microtox *Diluent* is the preferred option if

31 The Microtox test does not simulate the precise conditions in local bodies of fresh water, because the test is run at a fixed salinity of 2%. The estimates of toxicity obtained by using clean *Diluent*, therefore, are more suitable as "standardized" toxicity values for a particular chemical, wastewater, sediment or similar solid material.

Pollutants already in the receiving water can add toxicity to that of the chemical or wastewater under investigation. In such instances, the standard uncontaminated *Diluent* would give an accurate estimate of the toxicity of an effluent, leachate, or elutriate, but not necessarily of the total impact in the site of interest.

the sample itself were essentially seawater, such as a seawater elutriate, or a saline leachate or effluent. Use of clean seawater as diluent is preferred since the bacteria generally produce significantly more light in natural seawater than in *Diluent*, which uses only sodium chloride, even if salinity of the *Diluent* is adjusted upwards to that of seawater.

It would be a much less desirable option to use Microtox *Diluent* for testing a sample that was essentially or predominantly seawater, such as a seawater elutriate. The greater light production in seawater compared to NaCl solution, would mean an imbalance in the test with regard to the "background" production of light in the various dilutions of sample. That problem would not be solved by raising the salinity of the *Diluent*, using MOAS or sodium chloride, upwards from 2% salinity to a standard seawater value such as 3.5%, or to the salinity of the sample. Such adjustment of salinity should be done, if this option were selected for some reason, but it would be preferable to use the option described in the previous paragraph, of replacing *Diluent* with seawater.

If the effluent, leachate, or elutriate were saline but below the salinity of seawater, the Microtox test should not be run at less than 2% salinity (an exception could be rough range-finding tests). Light emission drops off appreciably as salinity declines below 2% (Krebs, 1983). All test salinities could, however, be adjusted upwards to above 2% but below the salinity of full seawater,

preferably by using appropriate amounts of clean seawater. The guiding principle in any such test is that the strength of "natural" seawater should be constant in all cuvettes.

7.4 Observations and Measurements on Sample

Colour, turbidity, odour, and homogeneity (i.e., presence of floatable material or settleable solids) of the effluent, leachate, or elutriate sample should be observed at the time of preparing test solutions. Samples with an appreciable solids content should be analyzed for total suspended solids (APHA *et al.*, 1989). Precipitation, flocculation, colour change, odour, or other reactions upon dilution with water should be recorded.

If there is concern about interference with light transmission by elevated colour, solids, floating or emulsified materials, the test should use the colour-correction technique outlined in Subsection 4.1.1 and Section 4.9.

7.5 Endpoints and Calculations

Tests for monitoring and for compliance with regulatory requirements might be single concentrations with a control (Section 7.2). Endpoints for these tests would depend on the objectives of the undertaking, but could include arbitrary "pass" or "fail" ratings based on a selected percent reduction in light emission at a given concentration. Multi-concentration tests would have the normal endpoint of IC50 as in Section 4.5.

Section 8

Specific Procedures for Testing Samples of Receiving Water

Instructions for testing samples of receiving waters, in addition to those provided in Sections 4 and 5, are given here.

8.1 Sample Labelling, Transport, and Storage

Procedures for the labelling, transportation, and storage of samples should be as described in Section 7.1. Testing of samples should start as soon as possible after collection, preferably within 24 h, and no later than 72 h after sampling.

8.2 Preparing Test Solutions

Samples in the collection containers should be agitated before pouring to ensure their homogeneity. Compositing of sub-samples should be as described in Section 7.2. For samples with high suspended solids, the cautions of Subsection 4.1.1 should be observed. The pH and dissolved oxygen content of the sample should be checked with regard to the limits in Subsections 4.1.2 and 4.1.3.

8.3 Dilution Water

For fresh surface waters, the standard (Universal) procedure should be used. For samples of marine or estuarine surface waters, there are additional options.

If the sample of receiving water is itself seawater, *MOAS* would not be added to the sample. In such a case, the preferred option is to use clean "natural" seawater as the diluent, i.e., for the controls and for diluting

the samples. The rationale and approach is described further in Section 7.3.

If the receiving water is estuarine, its salinity must be measured and adjusted upwards, if necessary, to achieve at least 2‰ salinity for the test. This could be done with *MOAS* or crystalline sodium chloride (NaCl), if desired, but it would be highly desirable for the diluent to contain clean seawater adjusted to the same strength (salinity) that was in the sample. If the sample of receiving water had a salinity greater than 2‰ but less than that of full-strength seawater, the best option would be to use a diluent of clean seawater diluted to the same salinity as that of the sample. Again, the guiding principle in any such manipulation would be that each cuvette in the test should end up with the same amount of seawater, and the same amount of added NaCl if that is used.

8.4 Observations and Measurements on Sample

Observations of sample colour, turbidity, foaming, precipitation, etc. should be made as described in Section 7.4, during preparation of test solutions.

8.5 Endpoints and Calculations

Endpoints for tests with samples of receiving water should be consistent with the options and approaches identified in Sections 4.5 and 7.5.

Tests for monitoring and compliance purposes should normally include, as a

minimum, an undiluted portion of the sample (Subsection 4.8.3), and one or more control solutions. In instances where toxicity of receiving-water samples is likely, such as mixing zones, and information is desired about the improvements or further dilution necessary to permit satisfactory conditions, a test to determine the IC50

should be conducted as described under Universal tests, Section 4. Often, surface water samples will not be toxic enough to cause an IC50. The Microtox system can be used to categorize surface waters on the basis of light loss caused by 45% sample, the highest concentration used in the standard procedure (Kaiser *et al.*, 1988a; 1988b).

Section 9

Specific Procedures for Testing Samples of Sediment or Similar Solids

General instructions are given here for testing liquids derived from samples of sediment or similar solids such as soils, and for the "solid-phase" Microtox test. These are in addition to general instructions provided in Sections 4 and 5. In this section, the word "sediment" is used for convenience but should be taken to include other similar solid substances such as soils, and industrial or municipal sludges, which might contribute pollutants to surface waters or require testing for other reasons.

9.1 General Aspects of Procedure

General guidance is given here on application of Microtox for testing sediments and the water derived from sediments. It is not the purpose of this report to provide instructions for carrying out a field survey of sediments, sampling them, or obtaining liquids or extracting materials from them. Detailed guidance for the collection, handling, transport, storage, and manipulation of sediments is provided in Environment Canada (1993). This guidance document should be consulted and followed when collecting samples of sediment and preparing them for Microtox tests. Additional guidance on these topics is provided in recent books (Mudroch and Macknight, 1991), reviews (Geisy and Hoke, 1989; McLeay and Sprague, 1991), and United States standard methods (ASTM, 1991a, 1991b).

Microtox has generally been judged as a useful and rapid method for comparing toxicity of extracts of contaminated

sediments (Schiewe *et al.*, 1985). It has been recommended as one of four toxicity tests to include in a battery of sediment tests, on the basis of an extensive review of available tests (Geisy and Hoke, 1989). A survey of 50 sediments from the coast of Washington State showed that Microtox, oyster embryo, and amphipod tests yielded good agreement, although there was benefit in using a diversity of tests. Microtox detected toxicity in a greater number of sediments than did either of the other tests (Williams *et al.*, 1986; Becker *et al.*, 1990). Other sediment tests showed that Microtox and a toxicity test with echinoderm embryos were the most sensitive of seven sediment tests evaluated (Pastorok and Becker, 1989). Strosher (1984) tested 48 liquids overlying waste drilling muds, and concluded that bacterial luminescence tests were more sensitive, reproducible, quicker, and more economical for evaluating toxicity than were two kinds of tests with fish and one with germination of seeds.

Brouwer *et al.* (1990) used a solid-phase Microtox test to characterize sediments in the harbour of Hamilton, Ontario. They found that method to be more sensitive to hydrophobic toxicants such as polychlorinated biphenyl (PCB) than another Microtox method based on a liquid elutriate. Similarly, the solid-phase test proved more sensitive than the liquid-extraction test, to sediments from Halifax Harbour (Tay *et al.*, 1991), and several other industrialized Canadian sites (van der Geest, 1991).

9.1.1 Sample Labelling, Transport, and Storage

General procedures for the labelling, transportation, and storage of sediment samples should be as described in Section 7.1. Additional guidance is found in Environment Canada (1993). Temperature limits are those described in Section 7.1, and in particular, samples must not freeze or partially freeze, and must not be allowed to dry (Environment Canada, 1993; ASTM, 1991b).

For the liquids derived from sediments, containers and handling procedures should be the same as those given in Section 7.1 for elutriates. If a non-aqueous solvent has been used to extract substances, a glass container should be used to store the liquid, so that it will not be affected by the solvent, nor leach materials into the sample.

Testing of samples should start as soon as possible after collection. Solid-phase tests, or extraction procedures, should begin within two weeks of sampling, and preferably within one week. Testing must start no later than six weeks after sample collection.³² Testing of the liquid obtained from sediments must begin within 72 h of making such preparations, or as specified in a regulation or other designated procedure.

9.1.2 Preparing Sample

Depending on the nature of the sample and the objectives of the test, homogenization of samples might or might not require mixing before testing. If mixing is carried out, it must be thorough. Sub-samples (i.e., a

sample divided between two or more containers) must be mixed together. If further sample storage is required, the composited sample, or a portion of it, should be returned to the sub-sample containers and stored.

9.1.3 Observations and Measurements on Sample

Observations of the colour, turbidity, foaming, precipitation, etc. should be made on both the sediment and any liquid derived from it, as described in Section 7.4, during preparation of test solutions.

9.1.4 Control or Reference Sediments

One or more samples of control or reference (unpolluted) sediment must be assessed in the same manner as the sediment under investigation. Although the test procedures include a blank or control which does not contain material from the sediment being studied, experience indicates that such a control might not be sufficient for an acceptable evaluation of toxicity. Environment Canada and other laboratories have frequently recorded apparent toxic effects with unpolluted sediments in solid-liquid suspension tests (Section 9.3). Accordingly, one or more control or reference ("clean") sediments should be included as a sample, with each test of a sediment or series of sediments, to help establish a baseline or "normal" level.

It would be desirable to establish a standard, clean "reference sediment" for this purpose, or a series of reference sediments of differing characteristics which could be

32 The toxicity and geochemistry of contaminated sediments from Hamilton Harbour was reported to change with storage for longer than one week, although the data supporting that statement were not provided (Brouwer *et al.*, 1990). Testing within two weeks conforms with current standardization in U.S. procedures (ASTM, 1991b) and with Environment Canada's (1993) recent guidelines. A maximum permissible storage time of six weeks has been specified (Environment Canada, 1993) in view of practical difficulties for shorter times, including time required if initial chemical analyses are to be performed.

matched with those of the sediments being tested. It would also be desirable to test for significant differences among results for the reference and test sediments. Testing a control or reference sediment, or liquid extract from such a sediment, is not necessarily required in *Microbics* procedures, but is an integral part of the procedure in the present document. The control or reference sediment(s) should be similar in general physical and chemical characteristics to the sediment(s) being investigated. In particular, an attempt should be made to match the distribution of particle sizes and organic/inorganic balance (ASTM, 1991a; 1991b; McLeay and Sprague, 1991; Environment Canada, 1993).

There is no single procedure for making use of the results from the control or reference sediment. If it shows no toxicity, then results for the test sediment (i.e., the one under investigation) are accepted as valid. If the control (reference) sediment does show toxicity, no standard method appears to have been developed, as yet, to adjust the results for the test sediment.³³ Caution should be exercised in interpreting findings for the test substance. Tests of statistically significant differences could be carried out, with guidance from a statistician, if paired observations or replicate tests are available.

9.2 Testing Liquids Extracted from Sediment or Similar Solids

Toxicants from sediments or soils can enter an aqueous phase and affect organisms in surface water, and Microtox can be useful for testing such effects. The aqueous phase might be a liquid derived from a soil or sediment (e.g., pore water), or a liquid used

to treat the sample and extract potential toxicants (e.g., an elutriate). The procedures recommended in Environment Canada (1993) for extracting pore water, or for preparing an elutriate, should be followed in deriving these liquids.

A liquid obtained from sediment for toxicity testing would be expected to fall into one of four broad categories.

- (1) Pore water, i.e., that which fills the spaces between particles, and could exchange with the overlying water making up the lake, river, estuary, etc. It is normally obtained from a sediment by centrifuging or squeezing it (Subsection 9.3.2).
- (2) Water used to obtain an aqueous extract of substances from the sediment (i.e., elutriate), for example by shaking a sample with added clean water.
- (3) Microtox *Diluent* or other pure water with adjusted salinity, used to obtain an aqueous extract as in (2).
- (4) Solvents other than water (e.g., organic solvents), used to remove substances from the sample of sediment (Schiewe *et al.*, 1985; True and Heyward, 1990).

The water in the first three categories could be tested as a normal liquid sample, following the universal procedures given in Section 4. Particular attention would be required for any problems caused by colour or turbidity (see Subsection 4.1.1 and Section 4.9).

The preferred option for the fourth category, solvents, is to have the same concentration

33 Brouwer *et al.* (1990) incorporated such a control by expressing the light emission of the test sediment as a percentage of that from the control (reference) sediment. However, only one concentration of each was tested, and the technique does not appear to have been extended to a multi-concentration test.

of solvent in each of the cuvettes. The *Diluent* to be used in the test is brought to the same concentration of solvent as that in the highest concentration of sample that will be tested. The solvent effect, if any, should be the same in all cuvettes. Another option is to include a solvent control at the highest concentration of solvent represented in the test, as well as a control without solvent. It would be desirable to run a separate test to determine the IC50 of the solvent. Specific steps for testing, when organic solvents are involved, are available as a manuscript from Microbics (1990) and might be included in future manuals.

9.2.1 Preparing Test Substances

Compositing of "sub-samples" of liquid obtained from the sediment (e.g., successive extractions) should be as described in Section 7.2 and recommended in Environment Canada (1993). Sub-samples would not be composited if it were desired to ascertain the relative toxicity of successive extractions. For samples with appreciable colour or turbidity, the procedures and cautions of Subsections 4.1.1 and Section 4.9 should be followed. The pH and dissolved oxygen content of the sample should be checked with regard to the limits in Subsections 4.1.2 and 4.1.3.

Once the liquid has been obtained, test concentrations are prepared in the standard manner (Subsection 4.2.2). As in testing effluents, leachates, and elutriates, the test could be a single-concentration (plus control) test for regulatory purposes, or a multi-concentration test to determine the IC50 (Section 7.2). The procedures for obtaining and testing liquid from samples of control or reference sediment should be identical to those for the test sediment.

9.2.2 Dilution Water

For freshwater samples derived from sediment, the standard (Universal) procedure should be used.

If the sediment samples are marine or estuarine, or saline waters are derived from them, there are additional options. If the water obtained from the sediment is essentially seawater, *MOAS* would not be added to the sample of water which is to be tested. The preferred option for diluent would be to use clean seawater for the controls and for diluting the samples. A less desirable alternative would be to adjust the salinity of the Microtox *Diluent* with *MOAS* to bring it to the salinity of the sample.

If the water derived from the sediment has a salinity lower than that of full-strength seawater, i.e., similar to estuarine water, its salinity should be measured, and adjusted upwards if necessary, to attain at least 2‰ salinity. If the sample of water has a salinity greater than 2‰ but less than that of full-strength seawater, the salinity of the diluent should be raised to the same salinity. The methods adopted to carry out these manipulations would be the same as outlined in Section 8.3.

9.2.3 Endpoints and Calculations

Endpoints for tests with liquids derived from sediment should be consistent with the options and approaches identified in Sections 4.5 and 7.5.

9.3 Solid-Liquid Suspension Test

A particular modification of the Microtox procedure, called the "solid-phase test" has been provided by Microbics and can be applied to sediments, sludges, soils, and similar substances. Since the procedure allows direct contact of the bacteria with the

solids of the sample, it is said to allow the assessment of toxic substances in or on the particles. In fact, liquid is added and the organisms are exposed to a suspension of the sediment; thus the test is also partly comparable to an aqueous extraction of the sample.

An early version of the procedure became available at the beginning of 1991 (Microbics, 1991a), and a more detailed draft was available later that year (Microbics, 1991b), and can be expected to undergo modification in details, as experience with the test accumulates. Therefore, only the general procedure is given here, and investigators are advised to obtain detailed procedures from the most recent instructions of *Microbics*.

To summarize the test, the phosphorescent bacteria are left in contact with saline aqueous suspensions of the sediment at various concentrations, for 20 minutes. The solid material is removed by filtration and the liquid is left for a further 5 minutes. Light emission from the liquid is then measured, and the IC50 is estimated.

9.3.1 Facilities

The test is designed to use the Model 500 *Analyzer* with analysis by a computer, so the general methods of Section 5 apply. The test can also be done, however, using the older Model 2055 *Analyzer*.

As well as the usual pipettes and flat-bottomed cuvettes, some special supplies are required:

Solid-Phase Diluent;
Solid-Phase Tubes; and
Solid-Phase Filter Columns.

9.3.2 Preparing Test Substance

After mixing, the sample of sediment is centrifuged or squeezed to remove free (interstitial) water³⁴ (ASTM, 1991b; Environment Canada, 1993). The solids are mixed again before use.

Ten *Solid-Phase Tubes* are readied in a rack and designated A1 to A5, and B1 to B5. Into tube B5, place 400 mg of the sediment which has been prepared by removing the water.

9.3.3 Beginning the Test

The steps are described precisely by Microbics (1991a) for a simple test with a series of nine concentrations and a control, and a synopsis is given here. Procedures using several controls, duplicate concentrations, etc. are given in Microbics (1991b).

- (a) Add 1.0 mL *Recon* to a cuvette in the *Reagent Well*.
- (b) Place cuvettes in wells A1 to A5, and B1 to B5.
- (c) Reconstitute a vial of bacteria in the usual way (Subsection 4.3.1 (a)), using the *Recon* from the *Reagent Well*, and return the *Reconstituted Bacteria* to that well.
- (d) Pour the *Reconstituted Bacteria* into a bottle of *Solid-Phase Diluent*, and mix well.

34 This water could be tested as described in Section 9.2. Some trials by Canadian laboratories indicate that the solid-phase Microtox tests show greater toxicity than is obtained with Microtox tests on the pore water obtained by centrifuging (Tay *et al.*, 1991; van der Geest, 1991).

- (e) Add 2.0 mL of the mixture from (d) to each of the *Solid-Phase Tubes* in a rack (Subsection 9.3.2) except the tube which contains sediment.
- (f) Set a timer for 20 minutes, and add 4.0 mL of the mixture containing bacteria from (d) to the *Solid-Phase Tube* B5 which contains sample, and mix well by shaking and pipetting.
- (g) Transfer 2.0 mL from Tube B5 to B4, then 2.0 mL from B4 to B3, and similarly in succession to B2, B1, A5, A4, A3, and A2, but not to A1. Each tube is mixed thoroughly before transferring from it. Discard 2.0 mL from Tube A2, so that each tube contains 2.0 mL. The highest concentration of solid substance is 10% in Tube B5, running downwards in two-fold dilutions to 0.039% in Tube A2, and zero in Tube A1.
- (h) At the 20-minute signal, filter contents of each tube in turn by pushing a filter column down in the tube.³⁵ Start at zero concentration (A1) and move upwards in concentration (ending at B5). Each time, transfer 0.5 mL of the liquid filtrate to the cuvette with the corresponding number.
- (i) Allow the liquid to stand for a further five minutes. Set the *Analyzer* ("Set" button)

on the liquid in cuvette A1, the control, and read the light emission. Read the emission from liquid in other cuvettes from low concentration (A2) to high (B5). Record the readings or have them recorded by the computer.

- (j) Repeat the procedure with the sample(s) of control or reference ("clean") sediment.

9.3.4 Endpoints and Calculations

Calculation of the IC50 is carried out in the general fashion outlined in Section 4.5. This would normally be done by the *Microbics* computer program (Version 6.0), and a manual check made for absence of errors and for reasonable calculations.

Certain features of the test should be noted. There is no "time-zero reading" (I_0) taken for each cuvette. The reading for the control (blank) is taken as the time-zero reading for each concentration as well as being the control reading. Therefore, the "blank ratio" (Section 4.5 (a)) is always 1.0 and need not be estimated, if the calculations are being done by hand. Some earlier versions of the *Microbics* computer program were limited to a two-fold factor of dilution for concentration, and an investigator is advised to use Version 6.0 of the program, or a later version.

35 *Microbics* standard filtration method will probably be generally adopted. This technique is recommended to allow comparison of results from different laboratories using a standard method. It is reported by Brouwer *et al.* (1990), however, that centrifugation gave at least an order of magnitude more toxicity in a solid-phase test than did filtration at any pore size from 0.1 to 8 μm .

Section 10

Reporting Requirements

The test report should describe the materials and methods used, as well as the test results. The reader should be able to determine from the report whether the conditions and procedures make the results acceptable for the use intended.

Procedures and conditions that are common to a series of ongoing tests (e.g., routine toxicity tests for monitoring or compliance purposes), and consistent with specifications in this document may be referred to by citation or by attachment of a general report which outlines standard laboratory practice. Where choices exist, the approach selected should be specified. The general report should convey the procedural information included in Sections 10.2 to 10.4. An individual test report giving the findings should contain the information indicated in Sections 10.1 and 10.5. Specific monitoring programs might require selected items (e.g., procedures and results for tests requiring salinity adjustment) in the test report. Other details pertinent to the conduct and findings of the test, which are not conveyed by the reports, should be kept on file by the laboratory, so that the appropriate information can be provided if an audit of the test is required.

10.1 Test Substance

- (a) Sample type, source, and description (chemical, effluent, leachate, elutriate, receiving water, sediment, or other similar solid substance; sampling location and method; specifics of the

nature, appearance and properties, volume and/or weight).

- (b) Information on labelling or coding of the test substance.
- (c) Details on manner of sample collection, transport and storage (e.g., batch, grab or composite sample, description of container, temperature of sample upon receipt and during storage).
- (d) Identification of person(s) collecting and/or providing the sample.
- (e) Dates and times for sample collection and start of definitive test.
- (f) Any physico-chemical measurements on sample (e.g., chemical concentration, colour, suspended solids content).

10.2 Test Organisms

- (a) Batch or lot number of organisms used.
- (b) Date obtained and temperature of holding.

10.3 Test Facilities and Apparatus

- (a) Name and address of test laboratory.
- (b) Name of person(s) performing the test.
- (c) Model number of *Analyzer* and description of any non-standard items of apparatus.

10.4 Test Methods and Conditions

- (a) Brief mention of method used if standard [e.g., this document or Microbics (1988a; 1988b; 1989b)].
- (b) Dissolved oxygen of sample used for testing, before and after initial aeration if any. Statement concerning aeration of solutions before the test (if any, give rate, duration, manner of application).
- (c) Any other chemical analyses of stock or test solution, and analytical procedure(s) used.
- (d) Procedure used in preparing stock and/or test solutions of chemicals.
- (e) Details of any salinity adjustment of samples.
- (f) Sampling and storage details if clean seawater was used for dilution of samples.
- (g) Concentrations tested including controls, and number of cuvettes at each concentration.
- (h) Appearance of test solutions and any changes during test.
- (i) Temperature as monitored in the incubation section of *Toxicity Analyzer*.

- (j) The pH of sample and description of any pH adjustment.

- (k) Observation times during test.

10.5 Test Results

- (a) Results for range-finding test (if conducted).
- (b) Code used for identifying chart recording, if used.
- (c) Time-zero readings of light emission.
- (d) Percent light inhibition in each test solution (including control) at each observation time.
- (e) Results of "colour-correction" test (loss of light transmission caused by colour and other attributes of sample).
- (f) Any IC50s and their 95% confidence limits, with method of calculation and values for important intermediate variables.
- (h) The IC50 and 95% confidence limits for the reference toxicant(s) determined within one month of the test, or when a new batch of *Bacterial Reagent* was first used, together with the geometric mean value (± 2 SD) for the reference toxicant as derived previously at the laboratory.

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Appendix A

Members of the Inter-Governmental Aquatic Toxicity Group*

*Federal***

P. Wells
EP, Dartmouth, N.S.

P. Jackman
St. John's, Nfld.

K.G. Doe
Dartmouth, N.S.

W.R. Parker
Dartmouth, N.S.

S.J. Wade
Dartmouth, N.S.

J.D. Vaughan
Dartmouth, N.S.

N. Bermingham
Longueuil, Quebec

C. Blaise
Longueuil, Quebec

D. St. Laurent
Longueuil, Quebec

G. Elliott
Edmonton, Alberta

R.G. Watts
North Vancouver, B.C.

S.G. Yee
North Vancouver, B.C.

D.J. Moul
North Vancouver, B.C.

K.E. Day
National Water Research Institute
Burlington, Ont.

A.K. Kwan
National Water Research Institute
Burlington, Ont.

C. Boutin
Canadian Wildlife Service
Hull, Quebec

J. Osborne
Office of Waste Management
Hull, Quebec

M. Bonnell
EcoHealth
Hull, Quebec

D. MacGregor
Regulatory Affairs and Program
Integration Branch
Hull, Quebec

R. Scroggins
Technology Development Branch
Hull, Quebec

G. Sergy
Technology Development Branch
Edmonton, Alberta

* IGATG membership as of November, 1992.

** Environment Canada, Conservation and Protection.

Federal, Fisheries and Oceans Canada

R. Stevens
Oceanography and Contaminants Branch
Ottawa, Ont.

Provincial

C. Bastien
Ministère de l'Environnement du Québec
Sainte-Foy, Quebec

S.G. Abernethy
Ont. Ministry of Environment
Rexdale, Ont.

C.M. Neville
Ont. Ministry of Environment
Rexdale, Ont.

D.G. Poirier
Ont. Ministry of Environment
Rexdale, Ont.

I.R. Smith
Ont. Ministry of Environment
Toronto, Ont.

G. Westlake (Chairperson)
Ont. Ministry of Environment
Rexdale, Ont.

B. Bayer
Manitoba Ministry of Environment
Winnipeg, Manitoba

J. Somers
Alberta Environment Centre
Vegreville, Alberta

K. Smiley
Alberta Environment Centre
Vegreville, Alberta

S.H. Horvath
B.C. Ministry of Environment
Vancouver, B.C.

G. van Aggelen
B.C. Ministry of Environment
North Vancouver, B.C.

Appendix B

Conservation & Protection Regional and Headquarters Offices**Headquarters**

351 St. Joseph Boulevard
Place Vincent Massey
Hull, Québec
K1A 0H3

Atlantic Region

15th Floor, Queen Square
45 Alderney Drive
Dartmouth, Nova Scotia
B2Y 2N6

Quebec Region

1141 Route De L'Eglise
P.O. Box 10100
Sainte Foy, Québec
G1V 4H5

Ontario Region

25 St. Clair Ave. East, 6th Floor
Toronto, Ontario
M4T 1M2

Western and Northern Region

Room 210, Twin Atria # 2
4999 - 98 Avenue
Edmonton, Alberta
T6B 2X3

Pacific and Yukon Region

224 Esplanade Street
North Vancouver, British Columbia
V7M 3J7



Appendix C

Sample of Possible Format for Recording Results of Microtox Tests

This example is taken from Beckman (1982).

SAMPLE ID NO.: N/A COLLECTED BY: K.W.
 DATE: 4-22-81
 SOURCE: Baker Reagent ZnSO₄·7H₂O TIME: 1000

REPORTED RESULTS: TOXIC ☐ NON-TOXIC ☐ MAX Γ = N/A FOR CONCENTRATION = N/A
 GRAPH ☒
 CALC ☐ EC (MIN. °C) = ☐ % SAMPLE ☒ mg/L
 CONFIDENCE FACTOR = ☐ OTHER
 95% CONFIDENCE INTERVAL TO
 REMARKS:

SAMPLE DATA: SAMPLE TYPE (EFFLUENT, LEACHATE, ETC.): purified chemical
 DATE ASSAYED: 4 . 22 . 81 TIME:
 VISUAL COLOR: none CORRECTION REQUIRED: YES ☐ NO ☒
 VISUAL TURBIDITY: none SEPARATION REQUIRED: YES ☐ NO ☒
 INITIAL pH: 5.6 for 170 pH ADJUSTED TO: N/A WITH N/A
 OSMOTICALLY ADJUSTED WITH: MOAS ☐ DRY NaCl ☒ OTHER ☐
 PRIMARY DILUTION OF SAMPLE: 170 diluted 50 to 1
 OPERATOR: K.W. REAGENT VIAL LOT NO. M: 101289
 REMARKS: For mgZn²⁺/L, multiply EC value by 65.4 ÷ 287.4

TABLE OF OBSERVED AND CALCULATED RESULTS									
BLANK CUVETTE	FINAL ASSAY CONCENTRATION (SPECIFY UNITS)	INITIAL READING $\Gamma(0)$	FINAL READINGS			BLANK RATIO CHECK			
			5-MIN $\Gamma(5)$	15-MIN $\Gamma(15)$	30-MIN $\Gamma(30)$	B1: $R(5) =$			
B1	0 (BLANK)	90.3	79.2	72.3	61	.8771			
C1	0 (BLANK)	88.5	77.9	71.5	60.3	C1: $R(5) = .8802$			
SUMS OF READINGS		178.8 ^A	157.1 ^B	143.8 ^B	121.3 ^B	$I_c =$ N/A			
MEAN BLANK RATIOS			$\bar{R}(5) =$	$\bar{R}(15) =$	$\bar{R}(30) =$	$I_c =$ N/A			
$\frac{B}{A}$	$+$	$\frac{C}{A}$	$= \bar{R}(t) =$	.8786	.8043	.6784	$C_c =$ N/A		
SAMPLE CUVETTE	FINAL ASSAY CONCENTRATION (SPECIFY UNITS)	INITIAL READING $\Gamma(0)$	FINAL READINGS			$\Gamma(t, T)$ EFFECTS			
			5-MIN $\Gamma(5)$	15-MIN $\Gamma(15)$	30-MIN $\Gamma(30)$	$\Gamma(5)$	$\Gamma(15)$	$\Gamma(30)$	$\Gamma_c(L)$
B2	3.7 mg/L	90	68.7	57.1	46.7	.1511	.2676	.3074	
C2	3.7	87.1	66.8	54.9	43.4	.1456	.2760	.3645	
B3	11.1	90.2	63.3	36.4	15.7	.2520	.9930	2.898	
C3	11.1	85.8	61.1	34.4	15.1	.2338	1.006	2.855	
B4	33.3	85.9	46.5	13.6	4.2	.6231	4.080	12.88	
C4	33.3	90	47	14.0	5.0	.6825	4.170	11.21	
B5	100	81.4	24.8	4.8	1.6	1.884	12.64	33.51	
C5	100	85.1	24.9	5.0	1.8	2.003	12.69	31.07	
EC 50 (1-MIN, 16 °C)			BY GRAPH			50.0	11.0	6.4	
			BY CALCULATOR						



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Fertilization Assay Using
Echinoids (Sea Urchins
and Sand Dollars)

Report EPS 1/RM/27
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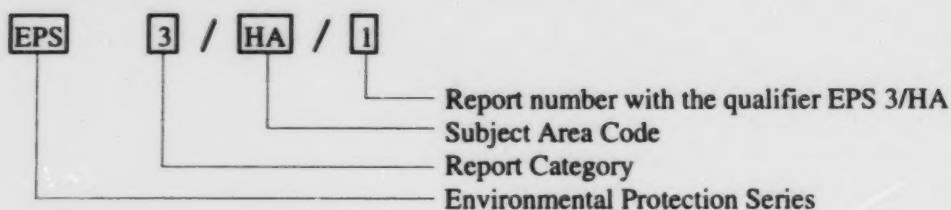


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Fertilization Assay Using Echinoids
(Sea Urchins and Sand Dollars)**

Environmental Protection
Conservation and Protection
Environment Canada

Report EPS 1/RM/27
December 1992

CANADIAN CATALOGUING IN PUBLICATION DATA

Main entry under title:

Biological test method. Fertilization assay using echinoids (sea urchins and sand dollars)

(Report ; EPS 1/RM/27)

Issued also in French under title: Méthode d'essai biologique. Essai sur la fécondation chez les échinides (oursins verts et oursins plats).

Includes bibliographical references.

ISBN 0-662-20430-1

DSS cat. no. En49-24/1-27E

1. Sea urchins -- Toxicity testing.
2. Sand dollars -- Toxicity testing.
3. Aquatic biology -- Environmental aspects.
- I. Canada. Environmental Protection Directorate. II. Canada. Environment Canada. III. Title: Fertilization assay using echinoids (sea urchins and sand dollars). IV. Series: Report (Canada. Environment Canada) ; EPS 1/RM/27.

QL384.E2B56 1993

593.9'5

C93-099487-6

Readers' Comments

Comments regarding the content of this report should be addressed to:

Richard Scroggins
Technology Development Branch
Environment Canada
Ottawa, Ontario
K1A 0H3

Garv Sergy
Technology Development Branch
Environment Canada
No. 210, 4999-98 Avenue
Edmonton, Alberta
T6B 2X3

Cette publication est aussi disponible en français. Pour l'obtenir, s'adresser à:

Publications de la Protection de l'environnement
Conservation et Protection
Environnement Canada
Ottawa (Ontario)
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Abstract

Methods recommended by Environment Canada for performing a sublethal marine toxicity test using gametes obtained from sea urchins or sand dollars are described in this report. In the test, sperm are exposed to the substance being tested. Eggs are then added, and the success of fertilization under continued exposure to the same concentration of test substance is measured. The endpoint is decreased success of fertilization, described in terms of the concentration estimated to cause a specified percent inhibition (IC_p), or the lowest-observed-effect concentration (LOEC) and no-observed-effect concentration (NOEC). The test is quick and is among the most sensitive of marine sublethal toxicity tests. Because the gametes and the success of fertilization usually represent a sensitive part of the life cycle, this assay should be considered as a powerful and meaningful sublethal test. The test may be run with five concentrations of test substance to determine the threshold of effect, or with one concentration as a regulatory or pass/fail test.

*Recommended species for use in this test are the green sea urchin (*Strongylocentrotus droebachiensis*) found on the Atlantic, Pacific and Arctic coasts of Canada, the Pacific purple sea urchin (*S. purpuratus*), or the eccentric sand dollar (*Dendraster excentricus*) from the Pacific. Provided that requirements for test validity are met, other local species may be used, or species from other geographic locations if a permit can be obtained to bring in specimens. Possible species to import include the Atlantic purple sea urchin commonly called *Arbacia* (*Arbacia punctulata*), and the white sea urchin from California (*Lytechinus pictus*).*

Procedures are given for holding adult echinoids in the laboratory, and obtaining sperm and eggs for a test. General or universal conditions and procedures are outlined for testing a variety of substances. Additional conditions and procedures are specific for testing sample(s) of chemical, effluent, receiving water, leachate, elutriate, or liquid derived from sediment or similar solid substance. Instructions are included for test facilities, handling and storing samples, preparing test solutions and initiating tests, specific test conditions, appropriate observations and measurements, endpoints and methods of calculation, and validation of the test.

Résumé

Le présent document expose les méthodes recommandées par Environnement Canada en vue de l'exécution d'un essai de toxicité sublétales en milieu marin sur les gamètes d'oursins verts ou d'oursins plats. Dans le cadre de cet essai, on expose d'abord le sperme à la substance étudiée. On ajoute ensuite des oeufs et on mesure le taux de fécondation, tout en maintenant l'exposition à une concentration constante de la substance étudiée. On obtient comme résultat une diminution du taux de fécondation que l'on exprime par la concentration estimée de la substance étudiée causant un pourcentage précis d'inhibition (CI_p), ou par la concentration minimale avec effet observé (CMEO) et par la concentration sans effet observé (CSEO). L'essai demande peu de temps et compte parmi les essais de toxicité sublétales en milieu marin les plus sensibles. La survie des gamètes et le succès de la fécondation étant des éléments essentiels du cycle de vie, cet essai constitue un instrument de mesure puissant et significatif de la subléthalité. On peut utiliser, dans le cadre de cet essai, cinq concentrations de la substance étudiée afin de déterminer la concentration seuil, ou encore une seule concentration, comme dans les essais réglementaires ou à résultat unique.

*Pour cet essai, on recommande d'utiliser les espèces suivantes : l'oursin vert (*Strongylocentrotus droebachiensis*) que l'on trouve sur les côtes atlantique, pacifique et arctique du Canada, l'oursin violet du Pacifique (*S. purpuratus*) ou le clypéaster excentrique (*Dendraster excentricus*) qui vit dans le Pacifique. Dans la mesure où elles satisferaient aux exigences relatives à la validité des essais, on peut également utiliser d'autres espèces locales ou encore des espèces d'autres pays si l'on obtient un permis d'importation. Parmi les espèces que l'on peut importer, mentionnons l'oursin violet de l'Atlantique, communément appelé *Arbacia* (*Arbacia punctulata*), et l'oursin blanc de Californie (*Lytechinus pictus*).*

Il présente également les méthodes permettant de conserver des échinides adultes en laboratoire et d'obtenir le sperme et les oeufs nécessaires à un essai. On y indique également les conditions et méthodes générales ou universelles qui doivent être mises en oeuvre pour réaliser des essais sur un large éventail de substances. D'autres conditions et méthodes sont propres à l'évaluation d'un ou de plusieurs échantillons de produits chimiques, d'effluents, de milieux récepteurs, de lixiviats, d'élutriats ou de liquides provenant de sédiments et de substances solides similaires. Le lecteur y trouvera des directives concernant les installations d'essai, la manipulation et le stockage d'échantillons, la préparation de solutions d'essai et la mise en route d'essais, les conditions d'essai particulières, les observations et mesures appropriées, les résultats d'essais, les méthodes de calcul et la validation de l'essai.

Foreword

This is one of a series of recommended methods for measuring and assessing the aquatic biological effects of toxic substances.

Recommended methods are those which have been evaluated by Conservation and Protection (C&P), and are favoured:

- for use in C&P aquatic toxicity laboratories;*
- for testing which is contracted out by Environment Canada or requested from outside agencies or industry;*
- in the absence of more specific instructions, such as are contained in regulations; and*
- as a foundation for the provision of very explicit instructions as might be required in a regulatory program or standard reference method.*

The different types of tests included in this series were selected on the basis of their acceptability for the needs of programs for environmental protection and management, carried out by Environment Canada. These reports are intended to provide guidance and to facilitate the use of consistent, appropriate, and comprehensive procedures for obtaining data on toxic effects of samples of chemical, effluent, leachate, elutriate, receiving water, and sediment or similar solid substance.

Mention of trade names in this document does not constitute endorsement by Environment Canada; other products with similar value are available.



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List of Abbreviations and Chemical Formulae

°C	degree(s) Celsius
cm	centimetre(s)
d	day(s)
DO	dissolved oxygen (concentration)
g	gram(s)
g/kg	grams per kilogram
h	hour(s)
HCl	hydrochloric acid
H ₂ O	water
ICp	inhibiting concentration for a (specified) percent effect
L	litre(s)
LOEC	lowest-observed-effect concentration
m	metre(s)
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
mm	millimetre(s)
mS	millisiemen(s)
MSD	minimum significant difference
N	Normal
NaOH	sodium hydroxide
NOEC	no-observed-effect concentration
SD	standard deviation
SI	Système internationale d'unités
sp.	species

TEC	threshold-effect concentration
TIE	toxicity identification evaluation
TM (™)	Trade Mark
µg	microgram(s)
µm	micrometre(s)
>	greater than
<	less than
≥	greater than or equal to
≤	less than or equal to
±	plus or minus
%	percentage or percent

Terminology

Note: All definitions are given in the context of the procedures in this report, and might not be appropriate in another context.

Grammatical Terms

Must is used to express an absolute requirement.

Should is used to state that the specified condition or procedure is recommended and ought to be met if possible.

May is used to mean "is (are) allowed to".

Can is used to mean "is (are) able to".

Might is used to express the possibility that something could exist or happen.

General Technical Terms

Acclimation is physiological adjustment to a particular level of one or more environmental variables such as temperature or salinity. The term usually refers to controlled laboratory conditions.

Compliance means in accordance with governmental permitting or regulatory requirements.

Conductivity is a numerical expression of the ability of an aqueous solution to carry an electric current. This ability depends on the concentrations of ions in solution, their valence and mobility, and on the solution's temperature. Conductivity in fresh waters is normally reported in the SI unit of millisiemens/metre, or as micromhos/centimetre ($1 \text{ mS/m} = 10 \text{ } \mu\text{mhos/cm}$). Conductivity is a standard method for measuring salinity, with the result read off as g/kg or "parts per thousand".

Dispersant is a chemical substance which reduces the surface tension between water and a hydrophobic substance (e.g., oil), thereby facilitating the dispersal of the hydrophobic substance throughout the water as an emulsion.

Emulsifier is a chemical substance that aids the fine mixing (in the form of small droplets) within water, of an otherwise hydrophobic substance.

Embryo means the undeveloped young animal, before it hatches from the egg.

Euryhaline is the ability to tolerate a wide variation in salinity without stress.

Flocculation is the formation of a light, loose precipitate (i.e., a floc) from a solution.

Gametes are the sperm or unfertilized eggs obtained from adult animals.

Larva is a recently hatched individual which has physical characteristics other than those seen in the adult of the species.

Minimum Significant Difference (MSD) means the difference between groups (in this fertilization assay, the difference in average percent fertilization) that would have to exist before it could be concluded that there was a significant difference between the groups. MSD is provided by Dunnett's multiple-comparison test, a standard statistical procedure.

Monitoring is the routine (e.g., daily, weekly, monthly, quarterly) checking of quality, or collection and reporting of information. In the context of this report, it means either the periodic (routine) checking and measurement of certain biological or water-quality variables, or the collection and testing of samples of effluent, leachate, elutriate or marine/estuarine receiving water for toxicity.

Percentage (%) is a concentration expressed in parts per hundred parts. One percent represents one unit or part of substance (e.g., effluent, leachate, elutriate, or receiving water) diluted with water to a total of 100 parts. Concentrations can be prepared on a volume-to-volume or weight-to-weight basis, and are expressed as the percentage of test substance in the final solution.

pH is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions on a scale from 0 to 14, with 7 representing neutrality, numbers less than 7 signifying increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.

Photoperiod is the duration of illumination and darkness within a 24-h day.

Precipitation means the formation of a solid (i.e., precipitate) from a solution.

Pre-treatment means, in this report, treatment of a sample or dilution thereof, before exposure of gametes.

Salinity is the total amount of solid substance, in grams, dissolved in 1 kg of water. It is determined after all carbonates have been converted to oxides, all bromide and iodide have been replaced by chloride, and all organic matter has been oxidized. Salinity can also be measured directly using a salinity/conductivity meter or other means (see APHA *et al.*, 1989). Salinity is reported here as g/kg. The term "parts per thousand" ($^{\circ}/_{\infty}$) is synonymous with g/kg.

Turbidity is the extent to which the clarity of water has been reduced by the presence of suspended or other matter that causes light to be scattered and absorbed rather than transmitted in straight lines through the sample. It is generally expressed in terms of Nephelometric Turbidity Units.

Terms for Test Substances

Brine is a solution of sea salts in water, in stronger concentration than in oceanic water. It can be obtained from filtered seawater by partial freezing and draining off the unfrozen liquid, freezing and partially thawing, or slow heating and evaporation. It can also be prepared by adding commercially available ocean salts to fresh or distilled water. The strength of brine used for this fertilization assay should be 60 to 90 g/kg.

Chemical is, in this report, any element, compound, formulation or mixture of a substance that might enter the aquatic environment through spillage, application, or discharge. Examples of chemicals that are applied to the environment are insecticides, herbicides, fungicides, sea lamprey larvicides, and agents for treating oil spills.

Control is a treatment in an investigation or study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific condition that is being studied. In an aquatic toxicity test, the control must duplicate all the conditions of the exposure treatment(s), but must contain no test substance. The control is used to determine the absence of measurable toxicity due to basic test

conditions (e.g., quality of dilution water, health of test organisms, or effects due to their handling).

Control/dilution water is the water used for diluting the test substance, or for the control test, or both.

Control sediment is a sediment that is essentially free of contaminants and is used routinely to assess the acceptability of a test.

Dechlorinated water means a chlorinated water (usually municipal drinking water) that has been treated to remove chlorine and chlorinated compounds from solution.

Deionized water is water that has been purified to remove ions from solution by passing it through resin columns or a reverse osmosis system.

Dilution water is the seawater or other saline water used to dilute a test substance in order to prepare different concentrations for the various toxicity test treatments.

Distilled water is water that has been passed through a distillation apparatus of borosilicate glass or other material, to remove impurities.

Effluent is any liquid waste (e.g., industrial, municipal) discharged to the aquatic environment.

Elutriate is an aqueous solution obtained after adding water to a solid substance (e.g., sediment, tailings, drilling mud, dredge spoil), shaking the mixture, then centrifuging or filtering it or decanting the supernatant.

Estuarine water is brackish seawater, residing in a coastal body of ocean water that is measurably diluted with fresh water derived from land drainage.

Leachate is water or wastewater that has percolated through a column of soil or solid waste within the environment.

Marine water is seawater residing in or obtained from the ocean, sea, or inshore location where there is no appreciable dilution by natural fresh water derived from land drainage.

Pore water is the water occupying space between sediment particles. The amount of pore water is expressed as a percentage of the wet sediment, by weight.

Receiving water is natural seawater (e.g., in a marine or estuarine waterbody) that has received a discharged waste, or else is about to receive such a waste (e.g., it is just "upstream" or up-current from the discharge point). Further descriptive information must be provided to indicate which meaning is intended.

Reconstituted seawater is fresh water to which commercially-available dry ocean salt has been added in a quantity that provides the salinity (and pH) desired for the water in the test.

Reference sediment is sediment collected from the field and selected to match the sediment being tested in properties such as particle size, compactness, total organic content, but "clean", i.e., without chemical contaminants. It is often selected from a site in the general vicinity of sediments being tested for toxicity, but uninfluenced by the source(s) of contamination.

Reference toxicant is a standard chemical used to measure the sensitivity of the test organisms in order to establish confidence in the toxicity data obtained for a test substance. In most instances a toxicity test with a reference toxicant is performed to assess the sensitivity of the organisms at the time the test substance is evaluated, and the precision of results obtained by the laboratory for that chemical.

Sediment is particulate material normally lying below water or formulated for experimental purposes.

Stock solution is a concentrated aqueous solution of the substance to be tested. Measured volumes of a stock solution are added to dilution water, to prepare the required strengths of test solutions.

Substance is a particular kind of material having more or less uniform properties.

Upstream water is natural seawater (e.g., in a marine or estuarine waterbody) that is not influenced by the effluent (or other test substance), by virtue of being removed from it in a direction against the prevailing current or sufficiently far across the current.

Wastewater is a general term that includes effluents, leachates, and elutriates.

Toxicity Terms

Acute means within a short period in relation to the life span of the organism, and would be within a few days for echinoids, which generally have a life span of several years, e.g., four to eight years for sea urchins. An acute toxic effect would be induced and observable within the short period.

Chronic means occurring during a relatively long period of exposure, usually a significant portion of the life span of the organism such as 10% or more.

Chronic toxicity implies long-term effects that are related to changes in such things as metabolism, growth, reproduction, or ability to survive.

Chronic value is the geometric mean of the NOEC and LOEC in tests which have a chronic exposure. See also *TEC* as a recommended term.

Endpoint means the variables (i.e., time, reaction of the organisms, etc.) that indicate the termination of a test, and also means the measurement(s) or value(s) derived, that characterize the results of the test (NOEC, IC_p, etc.).

IC_p is the inhibiting concentration for a (specified) percent effect. It represents a point estimate of the concentration of test substance that would cause a designated percent impairment in a quantitative biological function such as growth rate, or number of young per brood, compared to the control. For example, an IC₂₅ could be the concentration estimated to cause a 25% reduction in growth rate, relative to the control. This term should be used for any toxicological test which measures a quantitative effect or change in rate, such as growth, respiration, or reproductive rate. In the present echinoid test, unmeasured effects on sperm, on eggs, and on the fertilization process are given an overall assessment by the percent inhibition of fertilization. The term *median effective concentration* (EC₅₀) is not appropriate in tests of this kind because it is limited to quantal measurements, i.e., an estimate that 50% of the individual organisms exposed to that concentration would show a particular effect, while the other 50% would not show the effect. The echinoid fertilization assay does not completely conform with the requirements for an EC₅₀, because a major part of the exposure involves only the sperm and the chief effect might be on the sperm, but no direct quantal measurements are made of the numbers or proportions of sperm affected.

Similarly no specific observations are made on the eggs themselves at the end of their exposure. The measurements of reduced fertilization represent the end result of various effects on either sperm or eggs, or both. Accordingly, the measurements are best treated as quantitative observations, and described as the ICp for impaired fertilization.

Lethal means causing death by direct action. Death is defined here as the cessation of all visible signs of movement or other activity.

LOEC is the lowest-observed-effect concentration. This is the lowest concentration of a test material to which organisms are exposed, that causes adverse effects on the organism, effects which are detected by the observer and are statistically significant. For example, the LOEC might be the lowest concentration at which fertilization success differed significantly from that in the control. LOEC is generally reserved for sublethal effects.

NOEC is the no-observed-effect concentration. This is the highest concentration of a test material to which organisms are exposed, that does not cause any observed and statistically significant adverse effect on the organism. For example, the NOEC might be the highest tested concentration at which an observed variable such as fertilization success did not differ significantly from that in the control.

Static describes toxicity tests in which test solutions are not renewed during the test.

Sublethal means detrimental to a living organism, but below the level that directly causes death within the test period.

TEC is the threshold-effect concentration. It is calculated as the geometric mean of NOEC and LOEC. *Chronic value* or *subchronic value* are alternative terms that might be appropriate depending on the duration of exposure in the test.

Toxicity is the inherent potential or capacity of a substance to cause adverse effects on living organisms. The effect could be lethal or sublethal.

Toxicity Identification Evaluation (TIE) describes a systematic sample pre-treatment (e.g., pH adjustment, filtration, aeration) followed by tests for toxicity. This evaluation is used to identify the agent that is primarily responsible for

toxicity in a complex mixture. The toxicity test can be lethal or sublethal.

Toxicity test is a determination of the effect of a substance on a group of organisms, tissues, cells or other living material, under defined conditions. An aquatic toxicity test usually measures either (a) the proportions of organisms affected (*quantal*), or (b) the degree of effect shown (*graded or quantitative*), after exposure to specific concentrations of chemical, effluent, receiving water, leachate, elutriate, or interstitial water derived from sediment or similar solid substance. The echinoid fertilization assay must be considered a graded toxicity test since there is no measurement of the proportions of either sperm or eggs that are directly affected, only the degree of effect resulting from damage to one or the other.

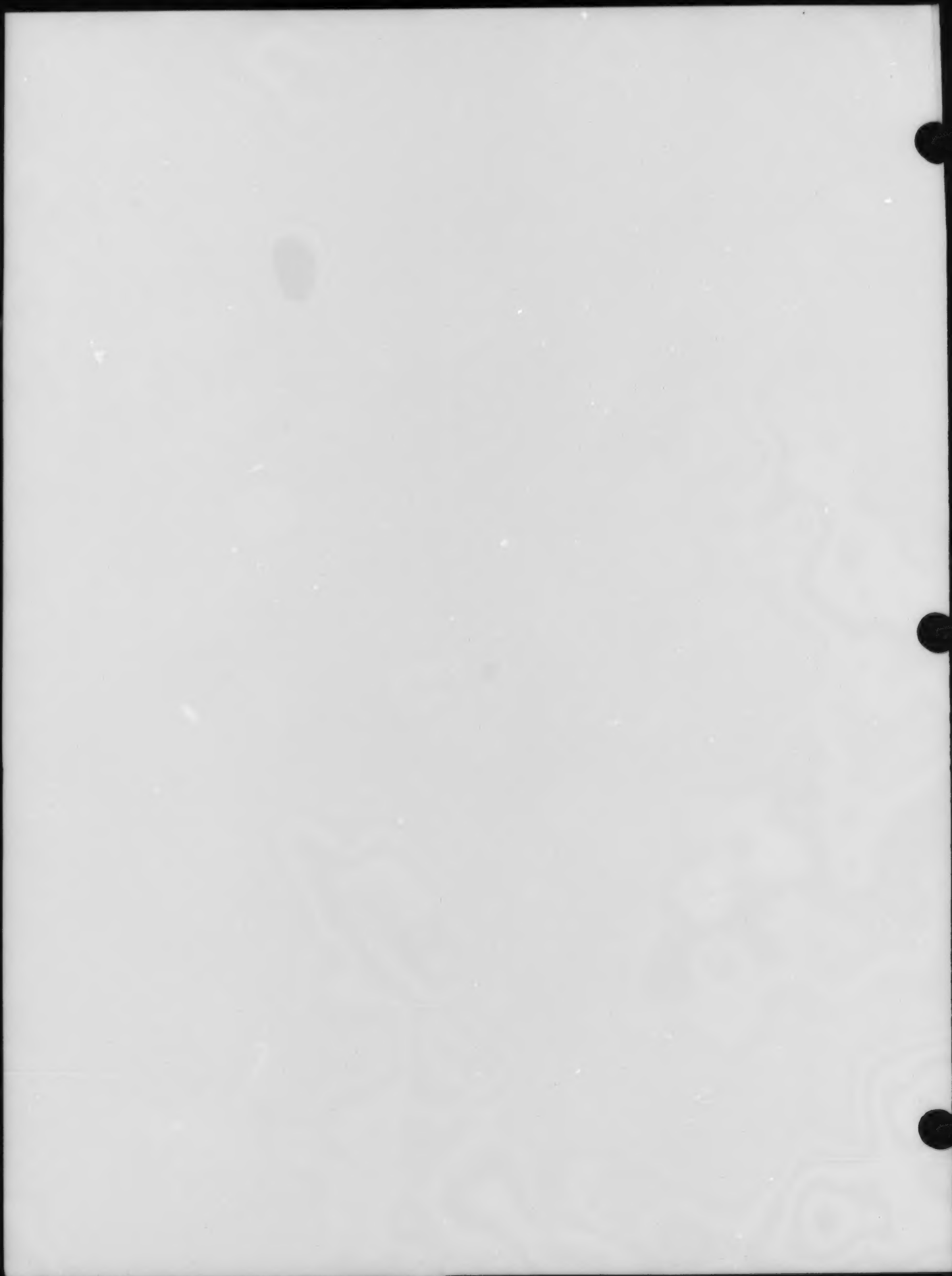
Acknowledgements

This document was co-authored by John B. Sprague (Sprague Associates Ltd., Guelph, Ontario) and Donald J. McLeay (McLeay Associates Ltd., West Vancouver, B.C.). It is based on pre-existing procedures of several authors, laboratories, agencies, and other groups in Europe, Japan, U.S.A., and Canada, and on a supporting review of relevant publications (Sprague and McLeay, 1991). Messrs. Gary A. Sergy and Richard P. Scroggins (Environmental Protection, C&P, Environment Canada) acted as Scientific Authorities and provided technical input and guidance throughout the work.

Members of the Inter-Governmental Aquatic Toxicity Group (IGATG, Appendix A) participated actively in the development and review of this document and are thanked accordingly. Special acknowledgement is made of the technical contributions provided by the IGATG Sub-Committee which was responsible for initial and final review: Gary A. Chapman (U.S. EPA, Newport, Oregon); Paul A. Dinnel (Univ. of Washington, Seattle, Washington); Timothy J. Hall (NCASI, Anacortes, Washington); John H. Himmelman (Laval University, Quebec); Emilia Jonczyk (Beak Consultants Ltd., Brampton, Ont.); Cathy A. McPherson (EVS Consultants, North Vancouver, B.C.); and IGATG members Kenneth G. Doe, Richard P. Scroggins, Gary A. Sergy, Graham C. van Aggelen, Peter G. Wells, and Stewart G. Yee. Substantial guidance came from laboratory tests on methodology by laboratories of Environment Canada (Atlantic, K.G. Doe; Pacific and Yukon, S.G. Yee), Beak Consultants Ltd. (Jennifer Miller, E. Jonczyk), and EVS Consultants (C.A. McPherson).

The following people provided information and many useful comments on final or early drafts: Brian S. Anderson (Univ. of California, Santa Cruz); Glenn F. Atkinson (Applied Statistics Div., Environment Canada, Ottawa); Robert S. Carr (U.S. Fish & Wildlife Service, Corpus Christi, Texas); Guy L. Gilron (Sentar, Surrey, B.C.); Larry R. Goodman (U.S. EPA, Gulf Breeze, Florida); Christopher W. Hickey (Water Quality Centre, Hamilton, N.Z.); Harold H. Lee (Univ. of Toledo, Toledo, Ohio); Richard Lloyd (Chelmsford, Essex, United Kingdom); Marian L. Nipper (Companhia de Tecnologia de Saneamento Ambiental, São Paulo, Brazil); James M. Osborne (C & P, Ottawa); Jerry F. Payne (Fisheries & Oceans, St. John's, Newfoundland); and Linda Porebski (C & P, Ottawa).

Photographs for front cover supplied by Mr. Stewart Yee, Aquatic Toxicology Laboratory, Pacific and Yukon Region, Environment Canada.



Section 1

Introduction

1.1 Background

Aquatic toxicity tests are used within Canada and elsewhere to measure, predict, and control the discharge of substances that could be harmful to aquatic life.

Recognizing that no single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection, the Inter-Governmental Aquatic Toxicity Group (see Appendix A) proposed the development and standardization of a set of single-species aquatic toxicity tests which would be broadly acceptable, and would measure different toxic effects using organisms representing different trophic levels and taxonomic groups (Sergy, 1987). A test based on fertilization success using gametes of sea urchins or sand dollars was one of several "core" aquatic toxicity tests which were selected to help meet Environment Canada's testing requirements.

Universal procedures for a fertilization assay with echinoid gametes are described in this report. Also presented are specific sets of test conditions and procedures, required or recommended when using the test to evaluate different types of substances (namely, samples of chemical, effluent, receiving water, leachate, elutriate, or interstitial water derived from sediment or similar solid substance) (Figure 1). Those procedures and conditions relevant to the conduct of the test are delineated and, as appropriate, discussed in explanatory footnotes.

In formulating these procedures, an attempt was made to balance scientific, practical, and financial considerations, and to ensure that the results will be accurate and precise enough for the majority of situations in which they will be applied. The authors assume that the user has a certain degree of familiarity with aquatic toxicity tests. Guidance regarding test options and applications is provided here. For regulatory use of the test, the choice of test options and applications is to be decided by the regulatory agency.

1.2 General Aspects of Echinoids and Their Use in Tests

Sea urchins and sand dollars belong to the Phylum Echinodermata and Sub-phylum (formerly Class) Echinoidea, and, therefore can collectively be called "echinoids". Other members of the phylum, not included in this test method, are the sea stars ("starfish"), brittle and basket stars, sea cucumbers, and crinoids or sea lilies and feather stars. The phylum has worldwide marine distribution and about 6000 living species are known. Seven species of sea urchins and three of sand dollars are commonly found in the coastal marine waters of Canada.

Echinoids and other members of the phylum are considered to be structurally advanced and complex invertebrates. They have many sophisticated features and many similarities to chordate animals including the basic pattern of embryonic development and some biochemical processes. The apparent radial arrangement of the body in five parts around a central axis is superimposed on a primary

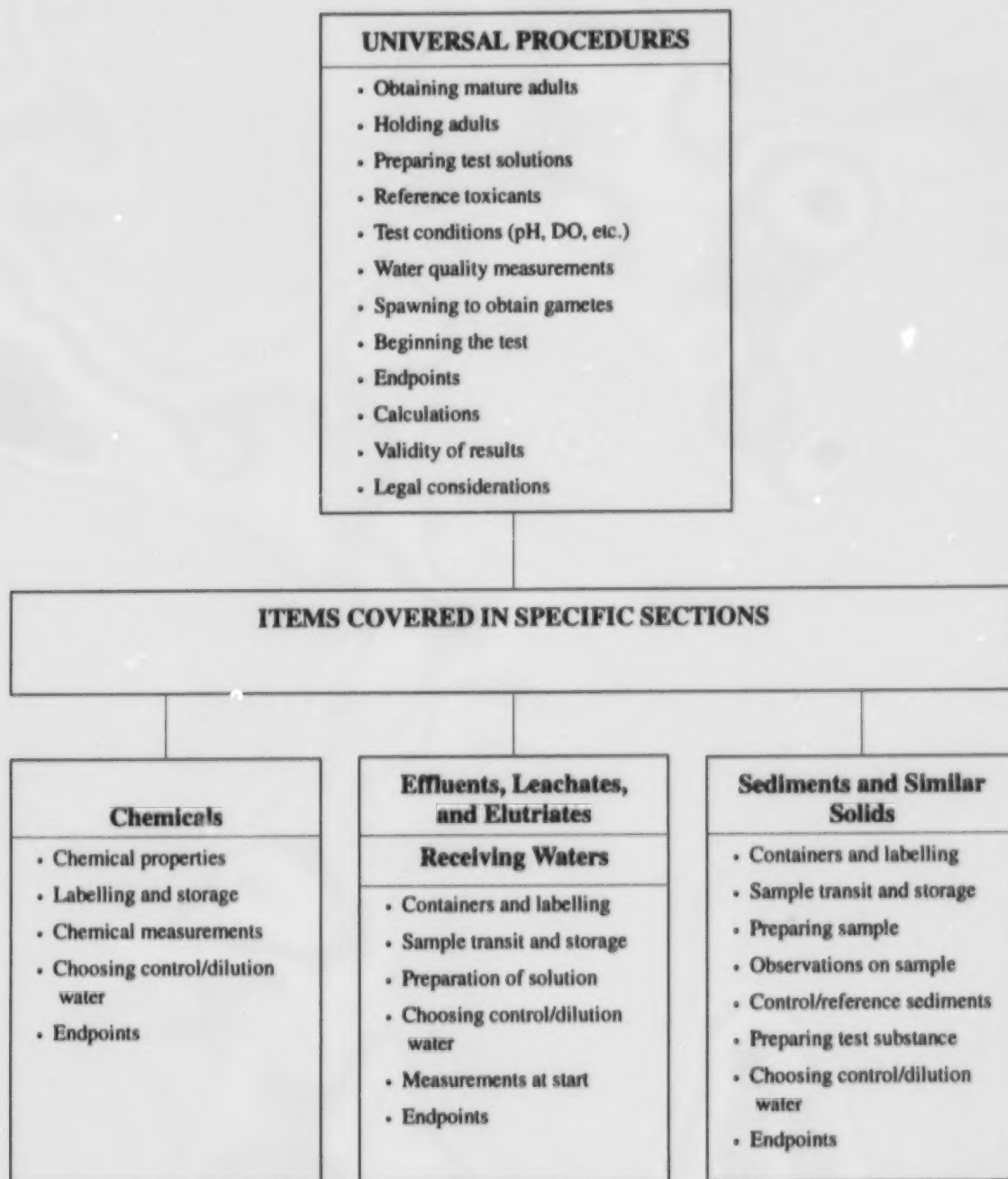


Figure 1

Diagram of Approach Taken in Delineating Test Conditions and Procedures Appropriate for Various Types of Substances

bilateral organization. There is a true internal skeleton covered by a thin epidermis. The skeleton is of small jointed calcareous plates, which in sea urchins and sand dollars are fused together into a solid *test*, or "shell", the latter term being used in this report for convenience. There is a well-developed coelom or internal body cavity, most of which surrounds the internal organs (Figure 2). Another part of the coelom is a tube-like *water vascular* ("hydraulic") system running to all parts of the body, used to manipulate small tube feet for locomotion, and to perform other functions.

Sea urchins are spherical and covered in spines, while sand dollars are flattened on the oral-aboral axis and generally disk-shaped (Figure 2). The oral surface is oriented downwards. A *peristomial membrane* surrounds the mouth in sea urchins, and injection of a chemical solution through that membrane and into the coelom is part of the procedure in these tests. For sand dollars, injection has to be through the mouth opening. The anus of sea urchins is on the aboral (upper) surface, but in sand dollars it is on the same surface as the mouth.

The sexes are separate but cannot be distinguished externally. There are large internal gonads (Figure 2) with outlets on the aboral surface, as five genital pores in urchins and four in sand dollars. One of the pores of urchins is in the *madreporite*, an obvious large plate of the shell, which is a terminus of the animal's water vascular system. The gametes are simply passed through the pores to the sea for fertilization.

The early development of sea urchins from egg to late larval stage ("pluteus" stage) is of great embryological interest, and more than 5000 papers were published on the topic by 1980 (NRC, 1981). This background has led

to the use of young stages of urchins in toxicity tests over many decades (Lillie, 1921; Drzewina and Bohn, 1926; Bougis, 1959), with a particularly thorough study of metal toxicity using fertilization in a sea urchin completed in the first quarter of this century (Hoadley, 1923). Both sea urchins and sand dollars are now frequently used as standard organisms in toxicity testing (reviewed in Dinnel *et al.*, 1987; 1988), and an extensive background of toxicological data has accumulated (Kobayashi, 1984).

The echinoid fertilization assay is sensitive. A major effect on egg fertilization, for example, was caused by municipal effluents at concentrations which were one-tenth of those causing 50% mortality of fathead minnows in a four-day test (Oshida *et al.*, 1981). It was the second to third most sensitive among six sublethal tests (marine and freshwater) used in an inter-laboratory survey of effluent toxicity in California (Anderson *et al.*, 1991). The 80-min echinoid fertilization assay was more sensitive to the effluent from a municipal waste treatment plant than were 48-hour tests with oyster and crab embryos and larvae (Dinnel and Stober, 1987). Variable results were obtained in a comparison of the toxicities of metal and organic compounds using the fertilization assay, a bacterial luminescence assay, and acute lethality tests with fish and crustaceans. Sometimes the echinoid test was one, two, or three orders of magnitude more sensitive, and sometimes an order of magnitude less sensitive (Nacci *et al.*, 1986). Results from echinoid fertilization assays were similar in sensitivity to those from embryo-larval tests with crab, squid, and fish, and were quite sensitive to metals, but much less so to pesticides than were tests of acute lethality using marine fish (Dinnel *et al.*, 1989). For pulp mill wastes, NCASI (1992) cites work of Johnson *et al.* (1990) that embryo-larval tests with oysters

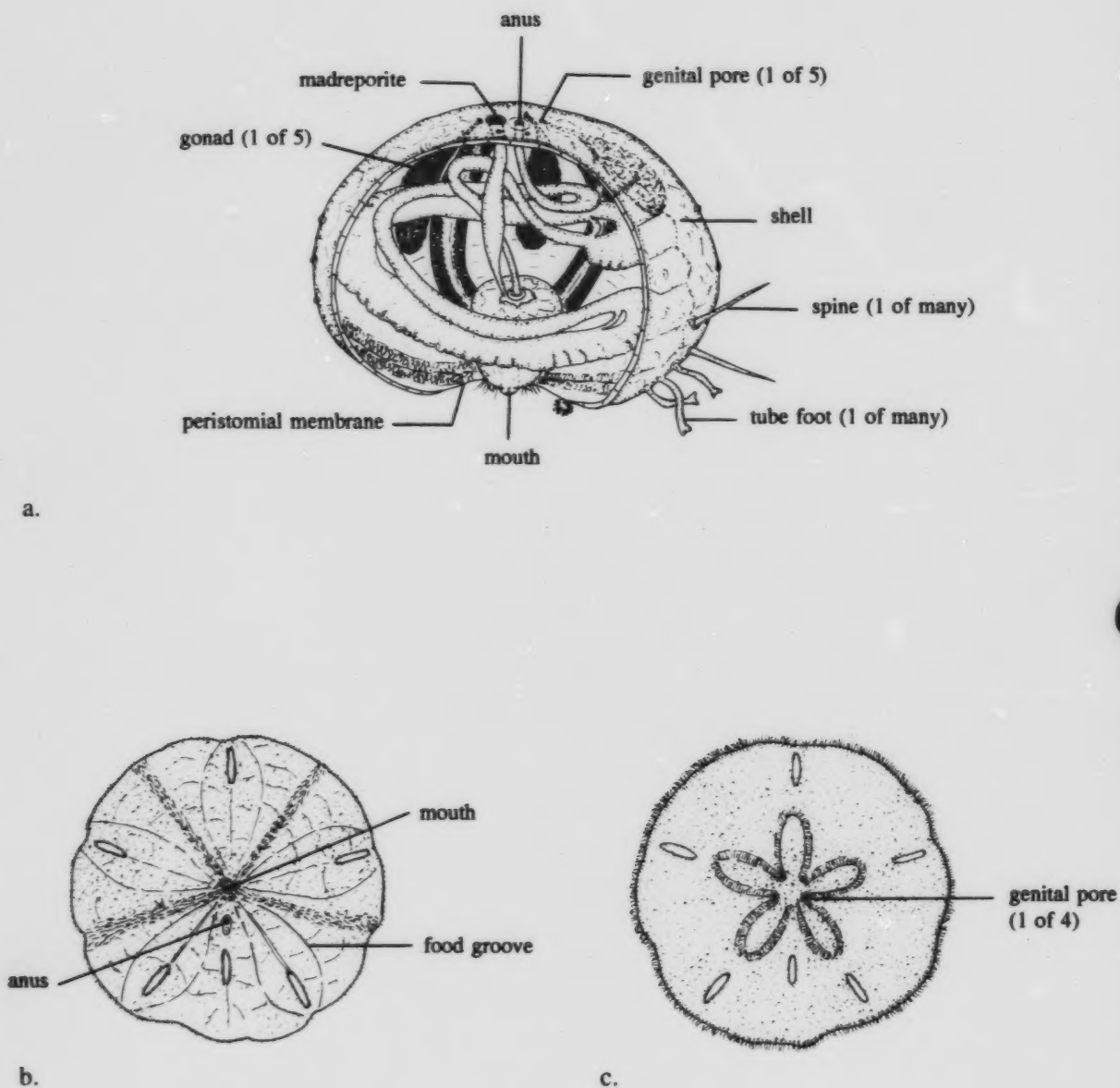


Figure 2

General Appearance of Echinoids.

a. Cut-away view of a typical sea urchin, *Arbacia*, showing location of genital pores on the aboral (upper) side. Only two or three of the numerous spines and tube feet are indicated. **b.** Oral side (normally down) of a typical sand dollar. **c.** Aboral side of a sand dollar showing location of the genital pores. (Drawings by M.A. White, after Storer *et al.* (1979) and Barnes (1974)).

were approximately an order of magnitude more sensitive than the echinoid fertilization assay. In turn, the echinoid assay was about as sensitive as a reproductive test using red alga, and was more sensitive, often by an order of magnitude, than other sublethal marine tests on growth and development of larval fish (silversides minnows and sheepshead minnows) or juvenile mysid shrimps (Schimmel *et al.*, 1989).

The fertilization assay is a sensitive sublethal test. Gametes and sperm of echinoids are either the most sensitive of the developmental stages, or are among the most vulnerable stages of the entire life cycle, when tested using various toxicants (Kobayashi, 1980; 1984). The fertilization assay is not a chronic test, however, because of its very short duration relative to the life spans of the species (some years). The fertilization assay described in this report is not intended to replace chronic toxicity tests using echinoids, because it might not estimate the effects of longer exposures. However, this test can be expected to yield results closer to such chronic tests than would conventional lethality tests with marine or freshwater species (e.g., Environment Canada, 1990a; 1990b).

Precision of the test appears to be satisfactory. The U.S. EPA (1988) determined that within-laboratory coefficients of variation for IC50s using reference toxicants and one species of sea urchin (*Arbacia punctulata*) were 30% to 48%. A coefficient of variation of 74% was found for IC50s of copper tested by six laboratories using four species of echinoids in an effluent testing program, compared to coefficients of 29% to 38% obtained with sublethal tests on single species (*Ceriodaphnia* reproduction, and early life stages of fathead minnows and oysters, Anderson and Norberg-King, 1991). In five

single-species comparisons among Canadian laboratories, the coefficients of variation were 62%, 65%, 75%, 82% and 110% for IC25s of copper (tests involved three species of sea urchins with total exposure times of 20 minutes). IC50s from the same tests showed lower coefficients of variation, with values 23%, 48%, 57%, 80% and 94% (Miller *et al.*, 1992). These interlaboratory coefficients, averaging 79% for IC25s and 63% for IC50s, are similar to the precision for chemical analyses, e.g., an average coefficient of 60% found in an interlaboratory comparison of chemical analyses of priority pollutants (Rue *et al.*, 1988). Recent unpublished results for an interlaboratory round-robin sponsored by the U.S. EPA are apparently similar, with coefficients of variation of 57% for 40-min fertilization assays and 86% for 80-min assays (NCASI, 1992).

The echinoid fertilization assay has been used in several Canadian aquatic toxicity laboratories, both governmental and industrial. Standard test methods have been described in British Columbia (B.C. MOE, 1990; van Aggelen, 1988), and by consulting companies (Beak, 1988; EVS, 1989). At the national level, a trial of methods has been carried out by certain Environment Canada laboratories (see Appendix B), under the sponsorship of a federal-provincial body (IGATG, 1991). Additional interlaboratory trials, involving federal, provincial (B.C. Ministry of Environment), and private testing facilities, were done the following year (Miller *et al.*, 1992). Echinoid tests have been reviewed and recommended by an Environment Canada scientist (Wells, 1982; 1984), but no standard method has been published by a Canadian federal government agency.

In the United States, several groups have provided methods for conducting sublethal

toxicity tests using echinoids. The United States Environmental Protection Agency has developed authoritative procedures for species of echinoids indigenous to their Atlantic (U.S. EPA, 1988) and Pacific (Chapman, 1991; 1992) coasts. Methods are also being developed by the American Society for Testing and Materials (ASTM, 1990). A critique of recent methodology has been provided by NCASI (1992), with special relevance to pulp and paper effluents. In addition, a number of consulting companies and other marine labs have written procedures for their own organizations (see Appendix C).

Numerous papers have been published by various authors and groups of authors who use standard techniques. Notable among these papers are those of Kobayashi, Dinnel and co-authors, and Pagano and fellow-workers. Some of their papers are in the reference list, and many others are in the bibliography (Appendix D).

There are several reasons for choosing an echinoid fertilization test as a method of assessing sublethal toxicity in Canadian marine locations. In general, the test is quick, sensitive, and relatively simple. Some advantages are:

- Much of the biology and life history of major species are documented.
- The organisms are commonly and widely distributed on the three Canadian coasts.
- Adult sea urchins and sand dollars are easily collected in shallow waters.
- Adults are readily held in the laboratory and conditions can be manipulated to lengthen their spawning season.

- Gametes of consistent quality and sensitivity can be obtained.
- Success of fertilization is a sensitive and fundamental sublethal effect to measure.
- The fertilization assay is rapid and economical because it is small-scale, easy to do, and uses ordinary facilities and supplies.
- Echinoid eggs are already haploid when released, unlike those of most animals, and so the need for a mandatory waiting period before use is avoided.
- The test has a relatively simple and objective endpoint.
- Echinoids are available worldwide, and are becoming frequently used as standard marine species for regulatory and research purposes. They can be easily shipped, and used at inland laboratories.

(NRC, 1981; Dinnel and Stober, 1985; Esposito *et al.*, 1986; Dinnel *et al.*, 1987).

In addition to general toxicity testing in a marine venue, the echinoid fertilization test would seem suitable for identifying the sublethally toxic components of complex effluents, using the "Toxicity Identification Evaluation" or TIE procedures described by the U.S. EPA (1991a, 1991b).

The purpose this "generic" report is to provide standardized Canadian methods for testing sublethal toxicity of various substances using echinoid gametes. Preferred choices are given among the alternatives available within a standard framework, for choice of species, exposure times, single-concentration (pass-fail) test versus multi-concentration test, test volumes, and type of water used for dilution and the

controls. If a Canadian regulatory test using echinoid fertilization success were to be promulgated, it would have a rigidly-defined method, selected from the alternatives presented in this report. The echinoid test procedures in existing documents vary in their coverage of endpoints, and of issues such as pH adjustment, alternative methods for various objectives, selection of control/dilution water, and how to deal with samples that contain appreciable solids or floating material. This report is intended for evaluation of sublethal toxicity in samples of chemical, effluent, leachate, elutriate, receiving water, and liquid derived from sediment and similar solid substances. The rationale for selecting certain approaches is given.

The method is meant for use with seawater-acclimated animals and seawater as the dilution and control water. Depending on the test objectives, this seawater may be reconstituted or natural, but should approach the salinity of full-strength seawater. Other tests, using freshwater-acclimated fish or other sensitive freshwater organisms, are available for evaluating the lethal and sublethal toxicity of chemicals or wastewaters that are destined for, discharged to, or within the freshwater environment (Environment Canada, 1990a; 1990c; 1992a; 1992b).

Section 2

Test Organisms

2.1 Species

The test may be done with any of the species listed below. Other native species may be used if they are found to be suitable.

Strongylocentrotus droebachiensis (O.F. Müller), the **green sea urchin**, a circumpolar species found on the Canadian Atlantic and Pacific coasts and across the Arctic Ocean to 80° N.

Strongylocentrotus purpuratus (Stimpson), called in this report the **Pacific purple sea urchin** (and commonly called the purple sea urchin), found on the Pacific coast of Canada and southwards to Baja California (Meinkoth, 1981).

Dendraster excentricus (Eshscholtz), a sand dollar of the Pacific coast of Canada and southwards, called in this report the **eccentric sand dollar**, a standard common name (Meinkoth, 1981).

Arbacia punctulata (Lamarck), called in this report **Arbacia**, although the common name of "Atlantic purple sea urchin" is sometimes used (Meinkoth, 1981). Found on the Atlantic coast of the United States from Cape Cod southerly into the Caribbean and Gulf of Mexico.

Lytechinus pictus (Verrill), the **white sea urchin**, found from southern California to Panama.

The first four species can be collected on one or more Canadian coasts. *Arbacia* and white sea urchins can be purchased from biological

supply houses and shipped to the test laboratory. Additional species might be available commercially or through interlaboratory arrangements, and might meet the needs of testing when local species are not in spawning condition.

All of these species have been listed as echinoids commonly used in the laboratory (NRC, 1981). Most of the species have been used frequently in toxicity tests (Appendix C). In general, toxicity results from fertilization assays using echinoids appear to be similar among species (Kobayashi, 1984; Nacci *et al.*, 1986). There might be small differences in sensitivity; the eccentric sand dollar appears to be about 1.4 times more sensitive to sodium dodecyl sulphate than the Pacific purple sea urchin, and 1.7 times more sensitive to bleached sulphite mill effluent than the green sea urchin (NCASI, 1992).

The common sand dollar, *Echinarachnius purma* (Lamarck), has not been used frequently in toxicity tests, and performed poorly in a recent multi-species interlaboratory evaluation of this echinoid fertilization assay (Miller *et al.*, 1992). Accordingly, the common sand dollar is not presently recommended for the test until research proves suitable. The circumpolar distribution of the common sand dollar, including its frequent occurrence along the Atlantic coast of Canada southwards to Maryland (U.S.A.), support further research with this species. Adult common sand dollars were successfully used for month-long toxicity tests in Newfoundland by Osborne and Leeder (1989). The effect

of growth-inhibiting chemicals and sediment contaminants on the early life stages of this sand dollar have been studied (Karnofsky and Simmel, 1963; Meador *et al.*, 1990).

Moving animals from one location to another marine location raises serious questions of introducing non-native species or transporting diseases and parasites. Any proposed procurement, shipment, or transfer of echinoids should be submitted for the approval of provincial or regional authorities. Provincial governments might require a permit to import organisms whether or not the species is native to the area, and movements of aquatic organisms might be controlled by a Federal-Provincial Introductions and Transplant Committee. Advice on contacting the committee or provincial authorities, and on sources of echinoids, can be obtained from the regional Environmental Protection office (Appendix B). Application for a permit to bring in animals must be made to the above-mentioned committee, to the appropriate provincial agency, or to the Regional Director-General of the Department of Fisheries and Oceans (DFO), depending on procedures in place locally.

2.2 Life Stage, Size, and Source

Mature and gravid echinoids should be obtained to provide the gametes. Adult sizes range from about 3-cm diameter upwards for the various species (Table 1); a common size-range for specimens in the laboratory is 5 to 6 cm.

All adults used to provide gametes for a test should be derived from the same population and source. The native species can be collected from clean-water coastal marine locations, some in shallow water at low tide,

or by diving. All species can also be purchased from biological supply houses.

The spawning seasons listed in Table 1 show that in a given location, tests could be carried out for much of the year by collecting sea urchins and sand dollars at appropriate times. The testing season could be lengthened by maintaining the adults at warm or cool temperatures to encourage early or late spawning. Canadian laboratories might be able to obtain gametes of the green sea urchin over most or all of the year by such changes in holding conditions (Wells, 1982; 1984). The other alternative would be to purchase species that had a suitable spawning time, from another location. It should be realized that animals from different sources and climatic conditions can show variations in timing and length of spawning season, or in the optimum temperature for bringing about spawning. Sea urchins that are spawned early in the season can sometimes provide gametes again in a month or six weeks if fed a proper diet (Dinnel and Stober, 1985). These sea urchins should be held in a separate tank after the first spawning.

Maturation should be checked before attempting to carry out a toxicity test with the gametes. Sperm and eggs obtained outside the main period of maturation can give poor fertilization rates and poor test results. Inspections for state of maturity require some experience on the part of the investigator, but can be assessed by spawning a sample of echinoids (Subsection 4.2.1) and examining the gametes. Mature sperm are minute and quickly become very active in seawater. Mature eggs rapidly become spherical in seawater. Immature eggs have a clear spot in the cytoplasm. Some adults could be sacrificed to examine the gonads, and to obtain gametes directly instead of by forced

Table 1 General Features of Echinoids Recommended for Use in Tests

Species	Spawning Season*	Maximum Diameter of Adult (cm) **	Holding Temperature in Laboratory (°C) ***
Green Sea Urchin	- generally April, but March to May at specific Canadian locations; a later cycle to June in the St. Lawrence estuary (January, June+)	8.3	9 , ≤12
Pacific Purple Sea Urchin	- generally January to May, optimally January to March for feral animals (December, June)	10	12 , ≤17
Eccentric Sand Dollar	- May through summer to October (February to December)	9	12 , ≤17
<i>Arbacia</i>	- June to August	5.1	15 , ≤22
White Sea Urchin	- March through summer to November	♂ 2.8 ♀ 3.2	15 , ≥8 ≤24

* Months in parentheses indicate possible extended spawning in the laboratory by holding at warm or cool temperatures. Information is taken from references used for Appendix C; from Meinkoth, 1981; NRC, (1981); Strathmann, (1987); Starr, (1990); and from information provided by reviewers listed in the Acknowledgments.

** The indicated sizes are the largest to be expected. Specimens held in the laboratory are often 5- to 6-cm diameter for most of the species.

*** Temperatures in bold type are recommended standard temperatures to obtain gametes in normal fashion, as derived from Appendix C and NRC (1981). Other temperatures could be used to speed or slow the maturation process, or the seasonal temperature of incoming natural seawater could be accepted. Maxima and minima are indicated in some cases.

spawning. In sea urchins, mature ovaries are coloured yellow to red depending on species, and testes are white.

2.3 Holding Adults in the Laboratory

2.3.1 General

Groups of male and female echinoids are held in tanks and used to provide gametes when required for a test. There is no particular limitation on time that the adults

may, or must be kept in the laboratory before providing gametes. Adult green and Pacific purple sea urchins and eccentric sand dollars have been fed and kept satisfactorily for months. A minimum holding time of 3 or 4 days is desirable, at the test temperature and in the water to be used for controls and dilution. The criterion of satisfactory holding conditions is the delivery of viable gametes that meet the needs of the test.

Echinoids should be handled with care and should not be subjected to sudden shocks or changes in holding conditions. In particular, large changes in temperature or hydrostatic pressure can stimulate spawning at a time that is not desired by the investigator (Dinnel and Stober, 1985). Some laboratories that use natural seawater without fine filtration have noticed mass spawning of sea urchins occurring at times of plankton blooms, and the phenomenon has been observed in Canadian waters (Starr, 1990; Starr *et al.*, 1990). In addition, spawning by individual animals can induce others to spawn, so such animals should be isolated immediately upon detection, to prevent mass spawning.

The recommended conditions for holding echinoids, summarized in Tables 1 and 2, are intended to allow some degree of flexibility within a laboratory, while at the same time standardizing those elements which, if uncontrolled, might affect the health of animals or viability of their gametes. Recommended conditions have been drawn, in general, from Appendix C. Further details and rationale are given in some of the publications included in Appendix C, and in the References, particularly ASTM (1990), U.S. EPA (1988), NCASI (1991), and papers of Dinnel and colleagues listed in the References and in the Bibliography of Appendix D.

2.3.2 Holding Containers

Adults may be held in aquaria, troughs, or tanks made of nontoxic materials such as glass, stainless steel, porcelain, fibreglass-reinforced polyester, perfluorocarbon plastics (Teflon™), acrylic, polyethylene, or polypropylene. Tanks containing about 50 to 150 L of water, and fitted with a standpipe drain, are most commonly used. The holding tanks should be located away from any major physical disturbances and preferably in a location

separate from that used for testing. To help avoid undesired mass spawning, adults should be held in groups of 20 or fewer animals.

For sea urchins, the water depth should be ≥ 20 cm. For sand dollars, trays are frequently used, for example, 1×2 m with a water depth of 10 cm. There should be 2 to 3 cm of sediment or sand, rich in detritus including settled algal cells, on the bottom of containers used for sand dollars.

2.3.3 Lighting

For sea urchins, the strength of lighting and photoperiod do not seem to be of major importance, and a low intensity of normal laboratory lighting is customary. For sand dollars, overhead fluorescent lighting at the equivalent of bright office lighting encourages algal growth on the sediment, which can result in desirable nutritional self-sufficiency for the tray of sand dollars.

2.3.4 Water

The water in containers holding adults should be renewed continuously or periodically to prevent a buildup of metabolic wastes. The water may be either an uncontaminated supply of natural seawater or "reconstituted" seawater that has been previously demonstrated to consistently and reliably support good survival and health of echinoids. The water supply should be monitored and assessed as frequently as required to document its quality. Temperature, salinity, dissolved oxygen, pH, and the volume of flow to each tank should be measured, preferably daily. Assessment of other variables such as total dissolved gases, ammonia nitrogen, nitrite, metals, pesticides, suspended solids, and total organic carbon, should be performed as frequently as necessary to document water quality.

Table 2 Checklist of Recommended Conditions and Procedures for Holding Echinoid Adults

Source of adults	- collected from clean-water areas or purchased from supply houses
Water	- uncontaminated natural seawater or reconstituted seawater; flow-through or semi-static (e.g., once every 24 h) replacement; average salinity from 28 to 34 g/kg, and individual measurements not outside 25 to 35 g/kg; as a general guideline, volume of flow should provide 5 to 10 L/d for each animal and equal the volume of tank in 6 to 12 h
Temperature	- from 9 to 15°C depending on species, somewhat lower or higher to delay or speed spawning, see Table 1; rate of change from one temperature to another $\leq 3^{\circ}\text{C/d}$
Oxygen/aeration	- dissolved oxygen 80 to 100% saturation; maintained by aeration with filtered, oil-free air if necessary
pH	- within the range 7.5 to 8.5, in normal circumstances 8.0 to 8.2
Water quality	- monitor temperature, salinity, dissolved oxygen, pH, and flow to each holding tank, preferably daily
Lighting	- normal laboratory lighting at low intensity; not considered critical
Feeding	- for sea urchins: kelp, other macroalga, or romaine lettuce; for sand dollars: provide sediment with detritus and alga, use lighting to encourage growth of algae, and if necessary add cultured alga
Cleaning	- removal of old alga, fecal material, and debris, daily or as required, unless intended as food
Disease/mortality	- monitor mortality daily; it should be $\leq 2\%/d$ averaged over 7 d preceding collection of gametes; remove diseased or moribund animals; groups of diseased animals should be discarded

As a general guideline for the flow rate of seawater in "once-through" systems, the investigator should aim at providing 5 to 10 L/d or more for each organism held, and also at having a flow that equals the tank volume in 6 to 12 h. For static holding tanks, a similar and acceptable exchange rate would be replacement of most of the water on a daily basis. There is no apparent consensus for amounts of water and exchange times in the existing methods (Appendix C). Most methods do not specify the flow, and the few that do, range from a high rate of hundreds of litres per animal per day, with an inflow equalling the tank volume in a few minutes, to lower rates which equal the tank volume in about 5 h. NCASI (1991, 1992) uses seawater flows similar to those recommended here, with 7 to 14 L/d per sand dollar and flow that equals the tank volume in 1.3 to 2.7 hours.

The average salinity of the water should be 28 to 34 g/kg, preferably 30 to 32 g/kg. Extreme salinity values should not be less than 25 or more than 35 g/kg during holding of echinoids.¹

Water entering the containers should not be supersaturated with gases, as might occur if the water were warmed. If that is a valid concern, total gas pressure in the water should be checked frequently (Bouck, 1982).

Remedial measures must be taken (e.g., use of aeration columns or vigorous aeration in an open reservoir) if dissolved gases exceed 100% saturation.

If reconstituted seawater is to be used as dilution and control water (see Subsection 4.1.1 and Section 5.3), adults should be acclimated to that water for at least three days, immediately before they are forced to spawn. Holding in reconstituted seawater or in a limited seawater supply can require filtration and recirculation of water, or its periodic renewal in static systems; ammonia and nitrite should then be measured frequently to check that they do not reach harmful levels. Target values, recommended for the protection of aquatic organisms, are ≤ 0.02 mg/L of un-ionized ammonia and ≤ 0.06 mg/L of nitrite (CCREM, 1987).²

Reconstituted seawater can be prepared by adding brine, commercially-available dry ocean salts or salt, formulations (e.g. Instant Ocean™) to a suitable fresh water, in quantities sufficient to provide the desired salinity. If ocean salts are used, the suitability and consistency among batches of a particular formulation of salts should be verified by testing, since some investigators feel that specific batches of sea salt can produce unwanted toxic effects or sequester test substances. If necessary, reconstituted

¹ The average salinity of world oceans is 34.7 g/kg, and varies from 32 to 37 g/kg except in the Arctic and nearshore areas where salinity can be less than 30 g/kg, or in hot areas of high evaporation rate, where salinity can be over 40 g/kg (Thurman, 1975; McCormick and Thiruvathukal, 1976). Echinoderms are well known to be osmo-conformers with narrow salinity tolerances. Himmelman *et al.* (1984) showed that at 24 to 25 g/kg compared to 30 g/kg, the green sea urchin had a decreased ability to right itself after being inverted, and that ability was a meaningful indicator of general health and physiological state of the animal. Of the existing methods for holding echinoids, those that recommend salinities do not specify values typical of the open ocean, but lower ones, mostly 27 to 30 g/kg with extremes of 25 to 35 g/kg (Appendix C). The normal coastal ocean salinity and guidance from past success in holding echinoids has been used in the present report, particularly with regard to lower limits of salinity (see also Subsection 4.3.2).

² The recommended target values are criteria for fresh water; however, they should also be protective for marine animals. There are no well established criteria for ammonia in seawater although the freshwater objectives are very well documented. The recommended limit for nitrite is not likely to be reached in marine waters under usual circumstances.

seawater could also be formulated from its individual component salts, with various degrees of sophistication for special situations that might be encountered. In such cases, a specialized reference should be consulted (Bidwell and Spotte, 1985).

Ocean salts may be added to seawater, samples, or other liquids to increase the salinity to the level desired for testing. A preferred alternative is to prepare a brine (very saline water) and add that as required. A brine can be prepared by freezing seawater at -10° to -20°C , and collecting the high-salt water under the ice when it reaches the desired salinity of ≤ 100 g/kg. That is conveniently done in a polyethylene carboy with a bottom-draining spigot (Chapman, 1992). Alternatively, the seawater could be completely frozen, then partially melted, with collection of the brine which melts first. Brine can also be prepared by slow heat-concentration of seawater at about 40°C until salinity reaches a value in the range 60 to 90 g/kg. Higher temperatures and stronger final salinity have been found undesirable in this evaporation method; when the brine is diluted back to normal salinity for seawater, it does not produce satisfactory conditions for aquatic organisms (ASTM, 1990). The seawater should be filtered through a pore size $\leq 10\text{ }\mu\text{m}$ before the freezing or evaporation process. Brine has been stored successfully for a year (NCASI, 1992). Using a brine of 90 g/kg to adjust the test concentrations to 30 g/kg means that the maximum concentration of effluent (or other freshwater sample) that could be tested would be 67%.³ Adjusting higher concentrations of a freshwater sample

to 30 g/kg would require the use of ocean salts.

Sources of water used for preparing reconstituted seawater may be deionized water, distilled water, an uncontaminated supply of groundwater or surface water, or dechlorinated municipal drinking water. If municipal or natural freshwater sources are used, this water should also be chemically assessed as appropriate to document its quality, for example the items listed at the beginning of this Subsection (2.3.4).

If municipal drinking water is to be used for preparing reconstituted seawater, effective dechlorination must rid the water of any harmful concentration of chlorine. The target value for total residual chlorine in water used for holding, control tests or dilution, is ≤ 0.002 mg/L (CCREM, 1987). Available chlorine as low as 0.05 mg/L is a potent spermicide for echinoids (Muchmore and Epel, 1973). Vigorous aeration of the water can strip out volatile chlorine gas. The use of activated carbon (bone charcoal) filters and subsequent ultraviolet radiation (Armstrong and Scott, 1974) is recommended for removing residual chloramine and other chlorinated organic compounds.⁴

2.3.5 Temperature

Echinoids may be held at standard temperatures (Table 1) or, if desired, at normal seasonal temperatures, e.g., using the temperature of the incoming natural seawater supplied to the laboratory. Temperatures should be adjusted to standard levels and held there for ≥ 3 d before the animals are

³ About 33% of the test solution would have to be brine, in order to attain the desired salinity.

⁴ Thiosulphate or other chemicals effective in removing residual chlorine from water should not be added to reconstituted seawater that will be used as control/dilution water in toxicity tests. Such chemical(s) could alter sample toxicity.

used to supply gametes for tests. A pre-spawning standard temperature of 9°C should be maintained for green sea urchins, 12°C for Pacific purple urchins and the two sand dollars, and 15°C for *Arbacia* and the white sea urchin.⁵

Groups of adults may be held for delayed spawning at temperatures that are lower than the seasonal norm for their habitat, the exact values varying with the species and the desired degree of delay. Similarly, temperature may be raised to encourage early development of gametes. Excessively high temperatures must be avoided, and recommended upper limits are 12°C for green sea urchins, 17°C for Pacific purple urchins and eccentric sand dollars, 22°C for *Arbacia*, and 24°C for white sea urchins, a species which should not be held at less than 8°C. Water temperatures may be changed to the desired value at a rate not exceeding 3°C per day.

2.3.6 Dissolved Oxygen

The dissolved oxygen (DO) content of the water within holding containers should be 80 to 100% of air saturation. If necessary to achieve that, mild aeration of the water should be carried out using filtered, oil-free compressed air. Such aeration through a commercial aquarium airstone also assists in mixing the water. Overly vigorous aeration should be avoided.

2.3.7 pH

The pH of water used for holding adults should normally be in the range 8.0 to 8.2, and must be within limits of 7.5 to 8.5. The average pH of ocean waters is 8.1 (Thurman, 1975) and seawater has a strong buffering

capacity. Coastal waters have a lower salinity than the open ocean, however, and some variation occurs from runoff of fresh water. Uncontaminated seawater is normally within the range of 7.5 to 8.5, whether it is brackish or full-strength, although the extremes of that range would be unusual. Existing methods for toxicity tests with echinoids do not give recommendations for the pH of water used to hold adults (Appendix C).

2.3.8 Feeding

Sea urchins are to be fed with kelp or macroalga (*Laminaria*, *Nereocystis*, *Macrocystis*, *Egregia*, *Hedophyllum*) or, alternatively, with romaine lettuce. Food should be added frequently enough (weekly, daily) that it is always available to the urchins, and old or decomposing food should be removed. Sea urchins have been held in the laboratory for years using macroalgae. The brown alga *Fucus* has been recommended as food (EVS, 1989) and also recommended against use (Dinnel *et al.*, 1987). The green sea urchin in Newfoundland eats *Fucus* and other brown alga such as *Alaria esculenta* as a major component of diet (Himmelman and Steele, 1971). The apparent feeding preference of the sea urchins being held should guide the investigator on use of *Fucus* and other potential food.

Sand dollars normally ingest particles selectively from the bottom and make use of the organic detritus available to them, including microalgae. For this reason, the natural and uncontaminated sediment used on the bottom of containers holding sand dollars should contain such detritus, and

⁵ At the beginning of the spawning season, or just before that season, it might be desirable to keep organisms at somewhat lower temperatures than indicated, to prevent spawning. For example, white sea urchins obtained in March might be held at 12°C rather than 15°C until spawning is desired. Advice should be sought from the people collecting at a particular site.

especially, settled plankton. Sand dollars have been said to require microalgae such as diatoms on the surfaces of sediment particles, and sufficient lighting can encourage growth of such algae on the sediment, increasing the success of long-term holding of the animals. Algae might be added to the sediment from a culture, if necessary (ASTM, 1990).

There are alternatives for feeding sand dollars which might sometimes be useful. Shredded eel grass (*Zostera* sp.) or even spinach could be added weekly, so that the animals can feed on the detritus (EVS, 1989). Flaked fish food may be used as a supplement (NCASI, 1991). However, any decomposing food in the tanks should be removed.

2.3.9 *Cleaning the Holding Containers*

Holding containers should be cleaned by scrubbing and rinsing before introducing a new batch of adults. Disinfectants may be used if it is desired to minimize the transmission of disease. Suitable disinfectants include those containing chlorinated or iodophore compounds or n-alkyl dimethyl benzyl ammonium chloride (e.g., Comet™, Ovidine™, Argentyne™, Roccal™). Disinfectants are toxic to aquatic animals, and traces could carry over on the tanks and affect the echinoids. If disinfection is used, each container must be thoroughly rinsed with the water used for holding.

When holding adults, the containers should be kept reasonably clean. Old macroalgae should be removed from urchin tanks, daily or as required. Periodic siphon-cleaning can be used in containers holding sea urchins,

and also in sand dollar containers for removing light detritus, fecal pellets, or replacing the sediment. Shell fragments could be left in tanks with sea urchins, since healthy urchins commonly cover themselves with such fragments.

2.3.10 *Disease and Mortality*

Adults should be inspected daily for signs of disease. Dead individuals should be removed immediately. In groups of animals which are intended to provide gametes, mortality should not exceed 2% per day, averaged over the seven days preceding collection of gametes.

Discard also, any moribund animals, sea urchins with significant loss of spines, and sand dollars with patches of fungus. Moribund sea urchins can usually be distinguished by lack of activity of the tube feet, inability to right themselves when turned over, and in particular by lack of adhesion to the substrate. Moribund sand dollars are usually distinguished by external appearance and activity. Such individuals often show patchy or overall pale colour as the epidermis degenerates, and do not rebury themselves. There is only weak activity of tube feet upon close inspection (magnifying glass or dissecting microscope), coupled with limpness of spines and pedicellaria (small pincer-bearing appendages among the tube feet). Dead sand dollars develop a coating of slime and often turn black.

Treatment of diseased adults with chemicals should not be attempted; it is strongly recommended that groups of animals showing a high incidence of disease be discarded.

Section 3

Test System

3.1 Facilities and Apparatus

Tests are to be conducted in a facility isolated from general laboratory disturbances. If a separate room is unavailable, the test area should be subject to minimal dust and fumes.

Construction materials and any equipment that might contact the test solutions or control/dilution water should not contain any substances that can be leached into the solutions or increase sorption of test substance (see Subsection 2.3.2). The laboratory must have the instruments to measure the basic variables of water quality (temperature, salinity/conductivity, dissolved oxygen, pH), and must be prepared to undertake prompt and accurate analysis of other variables such as ammonia.

All test solutions should be maintained within $\pm 1^\circ\text{C}$ of the desired temperature. This can be achieved using various types of equipment such as a temperature-controlled water bath in which test vessels are immersed.

3.2 Lighting

Normal laboratory lighting is satisfactory for the test.

3.3 Test Vessels

The three options for initial volume of test solution are 10.0 mL, 5.0 mL, and 2.0 mL; vessels must be suitable for containing the selected volume. These volumes span the usual range used in other written methods (Appendix C). The 10-mL volume is normally standard, and the smaller volumes are used for special purposes (see introduction to Section 4).

Borosilicate glass vials or tubes are to be used as test vessels. A capacity of about 20 mL is recommended for use with 10 mL of test solution, as is common practice (Appendix C, item 8). For smaller volumes of test solution, size of the vessels should be scaled down, to about double the volume of solution or somewhat more, e.g., vessels of 5 mL capacity for 2 mL of test fluid. However, larger vials may be used if desired, and vials up to 13 mL are sometimes used for 2 mL of test solution (Appendix C). A standard size should be selected and used within a laboratory.⁶

The vessels should have caps or some other seal, to avoid potential contamination from the air and loss of volatile components. The seal could be a sheet of plastic film which covers all the vessels in a test. The vessels should normally be of the disposable type, new and unwashed before use. An option is to reuse tubes after thorough washing and

⁶ Variations in size of test vessel might affect the results of the test through changes in relative depths, relative surface area of the fluid, and other variables, in ways that are as yet unrecognized. General agreement on exact sizes of test vessels does not appear to have occurred at the time of publication of this document. If such agreement develops in the future, investigators should harmonize with the trend, in order to minimize any potential effect of vessel size or proportion.

rinsing, but that technique has been known to result in measurable toxicity⁷, and is not recommended.

Considerable latitude is allowed in the design and shape of test vessels. For a given test, however, every treatment must use containers of identical type, size, and shape. Vessels of plastic are not recommended since there is evidence of deleterious effects on fertilization success (Dinnel *et al.*, 1987). In descriptions of existing procedures (Appendix C) the vessels are mostly disposable tubes of one kind or another, with caps, and made of borosilicate glass (such as PyrexTM). They are variously described as *scintillation vials*, *culture tubes*, *test tubes*, or simply as *tubes* or *vials*, and vessels of those designs would seem satisfactory, if of the appropriate size.

3.4 Control/Dilution Water

Depending on the test substance and intent (Sections 5 to 8), the control/dilution water may be "uncontaminated" natural seawater, reconstituted seawater, or a sample of receiving water collected "upstream" of the source of contamination. If receiving water is used, conditions for collection, transport, and storage should be as described in

Section 6.1. All marine waters used as a source of control/dilution water, including the laboratory supply of natural seawater, should be passed through a filter with a pore size of approximately 1 μm .

Salinity of control/dilution water should be 30 g/kg and must be in the range 28 to 34 g/kg. Lower salinities should be adjusted upwards with brine or sea salts (Subsection 2.3.4) and higher ones should be adjusted downwards with deionized water or uncontaminated fresh water.⁸

The pH of control/dilution water must be in the range 7.5 to 8.5, and should normally be 8.0 to 8.2. Those values would usually be obtained because of the natural buffering capacity of seawater. If not, adjustment should be made with acid or base (Subsection 4.3.4).

Control/dilution water must be adjusted to the test temperature before use. It must not be supersaturated with excess gases (see Subsection 2.3.4), and must contain dissolved oxygen at 90 to 100% of the air-saturation value before use. If necessary, achieve that level by aerating vigorously with oil-free compressed air passed through air stones.

⁷ Dinnel *et al.* (1987) tested different methods of preparing various kinds of vials. Fertilization was excellent in unwashed borosilicate tubes, and absent in acid-washed and rinsed glass tubes (i.e., very toxic). Fertilization was very poor in unwashed polystyrene and polyethylene tubes, and somewhat less toxic in washed plastic tubes, despite successful use of plastic tubes for other purposes such as tissue culture. Some laboratories have encountered sporadic toxicity in certain batches of new unwashed tubes, while most batches did not show toxicity.

⁸ Gametes of some or all of the test species would presumably tolerate salinities outside the recommended limits (see Subsection 4.3.2 and associated footnote), particularly higher salinities close to the oceanic average of nearly 35 g/kg. Salinities near 34 or 35 g/kg might, in fact, be less stressful than lower ones in the vicinity of 28 to 30 g/kg. The range of values recommended here considers the natural oceanic salinities and also the ranges used successfully in existing echinoid methods, i.e., salinities that are mostly near 30 g/kg (Appendix C).

Section 4

Universal Test Procedures

Procedures described in this section apply to all the tests of particular chemicals and wastewaters described in Sections 5, 6, 7, and 8. All aspects of the test system described in the preceding Section 3 must be incorporated into these universal test procedures. The summary checklist of recommended conditions and procedures in Table 3 includes not only universal procedures but also those for specific types of test substances.

There are some choices allowed within the general test procedures given in this report. Three options are available for duration of exposure. The shortest duration is a 10-min exposure of sperm, continued for an additional 10-min after eggs are added. That is the recommended standard exposure and it would minimize aging of gametes during a test or set of tests. The short exposure would also be most suitable for intensive programs involving many tests. For example, when attempting to identify toxic compounds in a complex effluent (TIE programs), successive manipulations of the effluent could be done before it aged appreciably.

A second option is a 20-min exposure of sperm plus 20 min of sperm plus eggs. That exposure might be used if it were desired to parallel certain existing methods or research results (Appendix C). The longest duration is 60 min of sperm plus 20 min of sperm plus eggs, an option that might be selected if maximum sensitivity were desired in the test. This longest exposure is also associated, however, with increased variation in results (see Subsection 4.2.4).

Three options are also available for the volume of test solution, which can be 10, 5, or 2 mL of each concentration of the sample. The 10-mL volume would be the usual standard choice and is preferred by Canadian investigators. The larger volume should be most convenient for manipulations by the operator and might improve the relative precision in handling small volumes. The smaller volumes require fewer adults to provide an assured supply of gametes, and can require less space in a water bath or constant temperature chamber. Small volume might be important for some investigations such as trials with pilot-plant outputs, perhaps as part of a TIE program.

4.1 Preparing Test Solutions

All test vessels, measurement devices, stirring equipment, and pails for transferring organisms must be thoroughly cleaned and rinsed in accordance with standard operational procedures. Control/dilution water should be the final rinse water for items which are to be used immediately in setting up the test; distilled or deionized water should be used as the final rinse for items which are to be stored after allowing them to dry.

4.1.1 Control/Dilution Water

The same control/dilution water must be used for preparing the control and all test concentrations. Each test solution should be well mixed with a glass rod, Teflon™ stir bar or other nonreactive device. Temperatures should be adjusted as required to within $\pm 1^\circ\text{C}$ of the test temperature. It might be necessary to adjust the salinity or

pH of the sample of test substance or the test solutions (see Subsections 4.3.2 and 4.3.4), or to provide preliminary aeration (Subsection 4.3.3).

If reconstituted seawater is to be used as the dilution and control water, the prepared solution should be homogeneous and aged for 1 to 2 weeks before use (Venables, 1986). If dry salts have been added, the water should be stirred gently for a minimum of 60 minutes (preferably with a magnetic stirrer) to ensure that the salts are in solution. Aging of reconstituted seawater should be done in a clean, covered vessel at the test temperature and with aeration. The salinity should be monitored during stirring and aging to make certain that the desired value is obtained and stable.

If natural seawater must be stored, it should be held at the test temperature or cooler, and used in three days or less.

The control/dilution water should be filtered to remove solids that might interfere with sperm counts, and the filtration is particularly important for natural seawater. A filter of pore size approximately $1.0\ \mu\text{m}$ is recommended, to remove most suspended solids (see also Section 6.2). Filtered water should be used in three days or less.

Receiving water may be used as control/dilution water to simulate local situations such as effluent discharge, a spill of chemical, or pesticide spraying. If that is done, a second control solution must be prepared using the laboratory seawater in which adults were kept (see Subsection 4.1.4). "Upstream" receiving water cannot be used, however, if it is clearly toxic and produces an invalid result in the control according to the criteria of this fertilization assay.⁹ In such a case, reconstituted seawater (Subsection 2.3.4) or the laboratory's natural seawater should be used as control/dilution water. The laboratory water could also be used if the collection and use of receiving water is impractical.¹⁰

4.1.2 Concentrations

For tests that are intended to estimate the NOEC/LOEC, the IC_p, or both, at least five concentrations plus a control solution (100% control/dilution water) are to be prepared. An appropriate geometric series may be used, in which each successive concentration is about 0.5 of the previous one (e.g., 100, 50, 25, 12.5, 6.3). Concentrations may be selected from other appropriate logarithmic series (see Appendix E). There is not usually a great improvement in precision of the test

⁹ Lower levels of contaminants, already in the receiving water, might not affect the controls by themselves, but could add toxicity to that contributed by the substance being tested. In such cases, uncontaminated dilution water (reconstituted or natural seawater) would give a more accurate estimate of the individual toxicity of the substance being tested, but would almost certainly underestimate the total impact at the site of interest.

If the intent of the test is to determine the effect of a specific substance on a specific receiving water, it does not matter if that receiving water modifies sample toxicity by the presence of additional toxicants, or conversely by the presence of substances that reduce toxic effects, such as humic acids. In the case of toxicity being added by the receiving water, it would be appropriate to include in the test, as a minimum, a second control of laboratory seawater or reconstituted seawater and, as a maximum, another series of concentrations using such clean water as diluent.

¹⁰ An alternative that could partially simulate receiving water would be to adjust the salinity (and perhaps the pH) of the laboratory seawater or reconstituted water to that of the receiving water. Depending on the situation, the adjustment might be to some particular seasonal value or mean. Adjustments could be made by methods mentioned in Subsection 2.3.4.

Table 3 Checklist of Recommended Test Conditions and Procedures**Universal**

Test type	- static; standard sperm exposure of 10 min, continuing with 10-min exposure of both sperm and eggs to allow fertilization; alternative exposures 20 + 20 min, or 60 + 20 min
Control/dilution water	- "uncontaminated" laboratory seawater; reconstituted seawater; "upstream" receiving water to assess toxic impact at a specific location, with additional control of laboratory seawater; filtered (1.0 μ m) before use; dissolved oxygen (DO) content 90 to 100% saturation at time of use; salinity 28 to 34 g/kg, preferably 30 g/kg, and pH 7.5 to 8.5, preferably 8.0 to 8.2
Organisms	- each replicate test vessel receives about 2000, 1000, or 400 eggs, depending on the selected volume of test solution; sperm:egg ratio is ascertained by trial as that which gives 90% fertilization under control conditions; this is normally in the range 200:1 to 2500:1, but could be much higher in some seasons
Replicates	- each concentration must have three replicates, and four are recommended
Vessel/solution	- standard volume of 10 mL test solution, with alternatives 5 or 2 mL; borosilicate glass vessels, capped or sealed
Temperature	- for the native species 15°C (green sea urchins, Pacific purple sea urchins, eccentric sand dollars), and 20°C for <i>Arbacia</i> and white sea urchins; range for individual test vessels $\pm 1^\circ\text{C}$ of desired temperature
Salinity	- as standard salinity 30 g/kg, limits 28 to 34 g/kg; each test solution in that range and also within 1 g/kg of the control; adjust as necessary with brine, sea salts, deionized or uncontaminated fresh water; samples of effluent, leachate, receiving water, and elutriate or other sediment-derived liquid should be adjusted to the salinity of control/dilution water; if it were desired to evaluate the total effect of a wastewater, including its salinity, an assay could be run without adjusting sample salinity, but a second adjusted test should be run, or salinity controls, or both
Oxygen/aeration	- no pre-aeration of aliquots of sample (e.g., effluent) or test solution unless DO is estimated to be <40% or >100% saturation in any concentration, in which case aerate an aliquot of the sample for ≤ 20 minutes at a minimal effective rate, before making up concentrations and starting the test

Table 3 Checklist of Recommended Test Conditions and Procedures (Cont'd)

pH	- regulatory or monitoring tests normally require no adjustment of pH of sample or solution; for other purposes, adjustment or a second (pH-adjusted) test might be required or appropriate; limits of pH 7.5 to 8.5, preferably 8.0 to 8.2, apply for minimizing direct effects of pH on the gametes, and maximizing the potential for detecting toxic chemicals
Lighting	- normal laboratory lighting
Observations	- percentage of fertilized eggs among 100 to 200 inspected microscopically for each test vessel
Measurements	- temperature, salinity, pH, and DO at start of exposure, in aliquots of test solutions for high, middle, low concentrations and control
Endpoints	- in multi-concentration tests, NOEC/LOEC for fertilization success; IC25 is a recommended option; in single-concentration tests, percent fertilization and whether significantly lower than control
Reference toxicant	- copper is recommended; determine NOEC/LOEC and/or ICp, at least monthly when tests are being run
Test validity	- average success of fertilization in control must be $\geq 50\%$ and $< 100\%$
Chemicals	
Solvents	- used only in special circumstances; maximum concentration 0.1 mL/L
Concentration	- measurement at start is recommended, in aliquots of high, medium, and low strengths and control(s)
Control/dilution water	- as specified and/or depends on intent; reconstituted seawater if high degree of standardization required; receiving water if concerned with local toxic impact; otherwise, the laboratory seawater
Effluents, Leachates, and Elutriates	
Sample requirement	- two litres should be adequate for the assay and for routine chemical analyses

Table 3 Checklist of Recommended Test Conditions and Procedures (Cont'd)

Transport and storage	- if warm ($>7^{\circ}\text{C}$), cool to 1 to 7°C with ice or frozen gel packs; transport in the dark at 1 to 7°C (preferably $4 \pm 2^{\circ}\text{C}$) using frozen gel packs as necessary; store in dark at $4 \pm 2^{\circ}\text{C}$; sample must not freeze; use in test should start within 24 h and must start within 72 h of sampling/extraction; extraction of sediment should occur within weeks and must occur no later than 6 weeks after sampling
Control/dilution water	- as specified and/or depends on intent; laboratory seawater or "upstream" receiving water for monitoring and compliance
Suspended solids	- standard treatment is filtration of sample at $5 \mu\text{m}$; if there is concern for loss of toxicity with the solids, a second test with unfiltered sample is an option
Receiving Water	
Sample requirement	- as for effluents, leachates, and elutriates
Transport, storage	- as for effluents, leachates, and elutriates
Control/dilution water	- as specified and/or depends on intent; if studying local impact use "upstream" receiving water
Sediment and Similar Solids	
Transport/storage	- temperatures as for effluents and leachates; test should start within 2 weeks and must start within 6 weeks
Preparing/testing	- aqueous samples derived from sediments should be treated as for effluents, leachates, and elutriates; solvent-based extracts should have balanced solvent concentrations; this is not a suitable assay for the solids themselves
Reference sediment	- parallel test with clean sediment of similar physicochemical properties
Control/dilution water	- as for effluents, leachates, and elutriates

from the use of concentrations closer together than those obtained with the 0.5 factor. In routine tests, concentrations should not be more widely spaced than those obtained using a factor of 0.3 (e.g., concentrations 100, 30, 9, etc.), because that leads to poor precision of the toxicity estimate. More widely spread concentrations might be used, however, if there is considerable uncertainty about the toxic levels.

Each desired concentration is prepared and the standard volume selected (10.0 mL, 5 mL, or 2 mL) is added to the replicate test vessels. These nominal concentrations of the solutions (or measured concentrations, see Section 5.4) are adopted as the concentrations of the test. The slight decrease in concentration upon addition of the aliquot of sperm suspension is neglected.¹¹ The nominal concentration during the exposure of sperm is adopted as the concentration of the entire test. There is a concentration decrease of about 9% in the final part of the test, after the suspension of eggs is added, but for purposes of characterizing the test, the initial concentrations for sperm exposure are used.¹²

In cases of appreciable uncertainty about sample toxicity, it is beneficial to run a

range-finding or screening test for the sole purpose of choosing concentrations for the definitive test. Conditions and procedures for running the test can be greatly relaxed. A wide range of concentrations (e.g., ≥ 2 orders of magnitude) should assist in selection for the full test.

Single-concentration tests could be used for regulatory purposes (e.g., pass/fail). They would normally use full-strength effluent, leachate, receiving water, elutriate or other liquid from a sediment or similar solid, or an arbitrary or prescribed concentration of chemical. Use of controls would follow the same rationale as multi-concentration tests. Single-concentration tests are not specifically described here, but procedures are evident, and all items apply except for testing only a single concentration and a control.

4.1.3 Replication

There must be at least three replicates prepared for each concentration including the controls. They are required for statistical analysis of results, specifically by Dunnett's test (Gulley *et al.*, 1989). It is recommended but not absolutely required that four replicates be used, because four are required for nonparametric statistical analysis, if results of the test do not satisfy requirements for normality and

¹¹ The actual concentrations to which the sperm are exposed would be 99% of the concentrations of the test solution, since 0.1 mL of sperm suspension is added to 10 mL of test solution, and proportional additions are made to tests of smaller volume. For example, if full-strength effluent were being tested in a vessel, the sperm would actually be exposed to $10/10.1 \times 100\% = 99.0\%$. No allowance is made for that slight difference.

¹² In some cases, at least, the sperm will be more sensitive than the eggs, and therefore the concentration during the initial sperm exposure can be the operative factor. For example, the toxic components of the effluent from bleached kraft pulp mills are reported to act primarily on the sperm rather than the eggs, in an echinoid fertilization assay (Cherr *et al.*, 1987). Kobayashi (1984) reviews his own work and that of other authors and concludes that male gametes are frequently "the most sensitive link in the success of fertilization and subsequent embryonic development".

homogeneity.¹³ The test must start with an equal number of replicates for each concentration including controls. If there is accidental loss of a replicate during the test, unbalanced sets of results can be analyzed with less power (Gulley *et al.*, 1989).

4.1.4 Controls

A control exposure is required for all tests, employing the same control/dilution water that is used to make up the test concentrations. The control must have the same number of replicates as in the rest of the test, i.e., at least three.

Salinity controls should be run if test salinity is, for any reason, outside the required range of 28 to 34 g/kg. If samples which were essentially fresh water (salinity ≤ 5 g/kg), were tested without adjusting salinity, salinity controls should be prepared by adding distilled water to a series of test vessels, at the same concentrations as used for the test liquid. The salinity controls indicate the effect of low salinity acting alone, but do not indicate any increased effect caused by interaction of low salinity with toxic substances in the sample (see Subsection 4.3.2).

If a solvent is used in testing a chemical that is sparingly soluble, then a "solvent control" must be run in replicate, and must contain the solvent at the highest concentration present in any test concentration.

If receiving water is used as the control/dilution water, a second set of controls must be run using the laboratory seawater that was used for holding the adults (see Subsection 2.3.4).

Additional kinds of controls are not required, but would improve the ability to judge quality of results. A "low-sperm" control would use only half the number of sperm in order to check for "over-sperming", which is a common imperfection in this assay. If the normal control achieved $>90\%$ fertilization and the low-sperm control was not 5% lower than the rate in the normal control, over-sperming is indicated, with associated poor sensitivity of the test. A "toxicant/egg control" or "egg blank" uses a high concentration of the toxicant, but no sperm; it can indicate whether the sample being tested causes false fertilization membranes. A "control blank" with eggs but no sperm can reveal accidental contamination of stocks of eggs with sperm (Chapman, 1991).

4.2 Beginning and Performing the Exposure

Semen containing sperm is collected from several echinoids by forced spawning. Semen from each individual is pooled before use. Eggs are collected, and pooled in the same fashion. Sperm are exposed to the test substance in each test vessel for either 10, 20, or 60 min. Then an appropriate number of eggs is added to each vessel, and exposure continues for 10 or 20 min to allow fertilization. Preservative is added to each vessel to end the exposure.

4.2.1 Collecting Gametes for the Test

The sperm should represent three or more male adult echinoids of the selected species, and the eggs should represent three or more adult females. Since it is possible that sperm or eggs from one adult might be particularly sensitive or particularly tolerant, an attempt

¹³ The requirements given here for replication are generally in line with those in the other methodology documents reviewed in Appendix C. Five of the methods recommend testing in triplicate, and four recommend "three or more" replicates, some acknowledging that four replicates would be required for nonparametric statistics. Five methods do not specify number of replicates.

must be made to achieve homogeneity of the experimental units, i.e., to avoid any differences among vessels that are related to the parent. The only practical way to do this is to pool the male or female gametes from different parents before transferring them to the test vessels. If gametes from three adults of each sex cannot be obtained, it is permissible to use fewer.¹⁴

The adults are stimulated to spawn by injecting potassium chloride.¹⁵ Sea urchins are injected with 1.0 mL of 0.5 M KCl through the peristomial membrane into the coelom (Figure 2). Sand dollars are injected with 0.5 mL of the same solution at an angle through the mouth. A tuberculin syringe with 25 gauge needle is satisfactory for this manoeuvre. An alternate method, often used with *Arbacia*, is stimulation of the shell for 30 seconds by electrodes supplied with 12 volts D.C.

The preferred technique for collecting semen from male sea urchins is called "dry spawning". One technique for dry-spawning male urchins is to place an individual in a beaker or petri dish rinsed with control/dilution water, with its aboral surface down. Semen is then collected from the bottom of the container (as opposed to from the surface of the animal). Another technique is to place the animal in a beaker with its aboral surface up, and barely cover it with control/dilution water. Extruded semen which accumulates on the animal's surface

by the pores is gathered with a micropipet, transferred to a small capped or covered tube, and stored on ice. Similar techniques may be used for collecting eggs from females, if desired, but they should be washed and stored as indicated below.

Male eccentric sand dollars produce insufficient volumes of sperm when spawned "dry". Sand dollars can be spawned in a minimal amount of seawater (5 mL); however, they should be suspended over the water column. (Experience indicates that sand dollars won't spawn if placed in a seawater-rinsed petri dish with their aboral surface in direct contact with the bottom of the dish.)

For the alternative "wet spawning" method, each sea urchin or sand dollar is placed aboral side down on a small beaker, 50 to 250 mL or other size as appropriate, filled to the brim with control/dilution water at the test temperature. After spawning is terminated, decant as much water as possible from the gametes.

If there is no spawning in 5 or 10 minutes, a second injection may be used. Semen or eggs should be produced by the adults in a steady stream, within half an hour of the final injection, as a maximum. Semen appears as a compact white string when shed into water, and eggs will appear as somewhat granular material, usually with a pastel colour (pinkish in sand dollars). Coloured

¹⁴ An optional procedure that could help ensure quality of the test would be to examine the gametes after they have been obtained from five or more individual adults, and to select and pool the batches that appeared most viable. The semen from each male would be stored separately on ice, a small portion would be diluted with control/dilution water on a microscope slide, and motility of the sperm would be judged. Sperm from the three most active samples would be pooled and used. Eggs from five or more individual females would be similarly examined under a microscope for poor quality as shown by small size, irregularity, or vacuolization. Eggs from three or more good batches would be pooled and used in the test. If good-quality gametes are not available from three adults, fewer batches should be used. "It is more important to use high quality [gametes] than it is to use a pooled population of [gametes]" (Chapman, 1992).

¹⁵ A solution of 0.5 M KCl is prepared by dissolving 3.75 g in 100 mL of distilled or deionized water.

products are sometimes extruded before or during the spawning, and should not be mistaken for gametes.

Collection of spawn should be terminated within 15 min of the start of steady spawning. Multiple collections of gametes from the same adult are normally pooled using a pipette. For manipulations of eggs, many investigators use a standard 1-mL plastic micropipette with 2 to 3 mm cut off by means of a scalpel, to provide a bore diameter of approximately 1 mm and reduce damage to the eggs.

Semen collected "dry" may be held on ice for 4 h before "activation" in seawater, then used in a test in the subsequent 30 to 120-min period.¹⁶ If sperm are collected in beakers of seawater, they should be used to start the test in a period ≥ 0.5 h to ≤ 2 h after collection is completed. In the interim, they are to be stored in a minimum amount of control/dilution water, on ice.

The collected eggs are washed three times by diluting with 100 mL of control/dilution water, mixing, settling for 10 minutes, and decanting. If pigmented substance is obtained with the eggs, it is important to

rinse them soon after collection, since the substance can be toxic with the Pacific purple sea urchin and perhaps with other species. Eggs may be held in the final addition of control/dilution water, at the test temperature, for 4 h until use. It is recommended that eggs be aerated gently during holding.

4.2.2 Preparing Standard Suspensions of Gametes

Semen is pooled to produce a concentrated suspension of sperm. If sperm were collected in beakers of water, pipet them from the bottom of the water and combine sperm from the various beakers. Semen should be transferred by drawing it slowly (without cavitation) into a micropipette (orifice ≥ 1 mm), and delivering by multiple expulsions and refills, to rinse it into the water receiving it.

Sperm density in the initial suspension is estimated with a hemocytometer or other counting cell under 400 $\times$ magnification.¹⁷ Dilute a small sample (0.1 to 1 mL) of the mixed suspension 100-fold to 10 000-fold (depending on concentration of sperm), using 10% glacial acetic acid made up with control/dilution water. Mix by inverting ten

¹⁶ Sperm commence a series of metabolic changes when exposed to seawater, enabling them to carry out fertilization. Following that activation, there is a period of an hour or less of vigorous activity, followed by gradual loss of vitality in the following 6 to 9 hours, the loss becoming more rapid at higher temperatures (Dinnel *et al.*, 1987). Some work shows a large increase in variability of test results if sperm are held in seawater before testing. Comparison of the toxicity of bleached kraft pulp mill effluent for eccentric sand dollar fertilization showed that "pre-activation" of sperm for 60 min in seawater, before the 80-min test, increased the coefficient of variation to 91%, from the value of 38% for sperm used without the pre-activation. Sensitivity of the test increased by a factor of 1.6 for pre-activation, i.e., a smaller relative change than the increase in variation (NCASI, 1992).

Activation of sperm can be delayed, and the useful period before starting a test prolonged by keeping the semen cool (on ice), in "dry" condition as released from the adult. The time limits for holding gametes, given in Subsection 4.2.1, are strongly recommended as a means of standardizing the test. Other times and techniques may, however, be used if it is demonstrated that fertilization rates of about 90% are normally obtained in the controls. Some investigators report that "dry" semen can be held satisfactorily on ice for longer periods, e.g., 8 hours for *Arbacia* (Carr and Chapman, 1992), but 4 hours is a more usual maximum (Chapman, 1992).

¹⁷ A very detailed explanation of the hemocytometer and its use for counting sperm is provided by Chapman (1992).

times and allow bubbles to clear for a minute or two. Add a drop of the mixture to the hemocytometer counting chamber and let the sperm settle for 15 minutes. Count the sperm in the middle 400 small squares. Calculate the number of sperm per mL in the initial suspension. This is done by multiplying: (dilution factor) $\times$ (number of sperm counted) $\times$ (hemocytometer conversion factor) $\times$ (conversion of mm³ to mL) $\div$ (the number of squares counted). For a standard hemocytometer (Neubauer), the formula becomes:

$$\text{No. sperm/mL} = 100 \times (\text{No. of sperm counted}) \div 4000 \times 1000 \div 400$$

Adjust the initial suspension of sperm to the desired concentration in a "standard sperm suspension", using control/dilution water.¹⁸ The concentration of this standard sperm suspension is determined by the sperm:egg ratio that is selected (Subsection 4.2.3).

An alternative counting technique that may be used, is to use turbidity as an indication of the number of sperm/mL, without a hemocytometer count. The advantage is a saving of time, since the turbidimetric measurement takes only one minute compared to 20 or 30 minutes with a hemocytometer (NCASI, 1992). That in turn allows tests to start sooner after collection of gametes. The concentrated collection of sperm is mixed with control/dilution water in a 1-cm spectrophotometer tube, just before starting the test. Standard turbidity meters designed for analysis of water samples may be used. NCASI (1992) reports that a range of 2.0 to 4.0 Nephelometric Turbidity Units

(NTU) usually yields the desired numbers of sperm. A count of 2.5 million sperm/mL would be associated with about 3.0 NTU for the eccentric sand dollar, and about 2.7 NTU for the Pacific purple sea urchin. The turbidimetric technique can have precision that is almost as good as that obtained by counting. NCASI (1992) found an average coefficient of variation (C.V.) of about 9% for repeated hemocytometer counts of single dilutions of sperm, and a C.V. of 12% for repeated hemocytometer counts of dilution to 5.0 NTU of sperm from three males. No evaluations of the turbidimetric method are available from other laboratories at the time of writing. The final criterion of whether turbidimetric assessment of sperm density was satisfactory would be the fertilization rate achieved in the control, during the test, compared to the optimum of 90% (Subsection 4.2.3), minimum of $\geq 50\%$, and maximum of $< 100\%$ fertilization (Subsection 4.5.1).

There are three options for initial test volume, the standard of 10 mL and of 5 or 2 mL. The concentrations of the gamete suspensions are the same for each. The amount of gamete suspension to be added is scaled down proportionally for the smaller test volumes. In the largest test volume (10 mL), there is 0.1 mL of sperm suspension added, and 1.0 mL of egg suspension. (See Table 4 for summary of numbers of gametes and volumes of gamete suspensions for the three sizes of test.)

The numbers of gametes and procedures are given here for a test with initial volumes of 10 mL. The required strength of the sperm suspension must be calculated first. About

¹⁸ The instructions for amounts of water used for the initial suspension are necessarily indeterminate. The particular technique used to collect semen will govern the concentration of sperm that is obtained in the initial suspension and the required dilution for a standard suspension. A given laboratory and investigator will normally develop standardized methods of collection and dilution that achieve somewhat predictable concentrations and dilutions that are satisfactory for counting.

2000 eggs are used in the 10-mL test, and the ratio of sperm to eggs is often in the range 50:1 to 2500:1 (Subsection 4.2.3), although it may sometimes be higher, to 20 000:1 or more. Within the range 50:1 to 2500:1, the required number of sperm would be from 100 000 to 5 million. Since 0.1 mL of the sperm suspension is added in the test, the concentration of sperm required in the standard suspension will usually be in the range one million to 50 million per mL.¹⁹

Calculations of proper dilution are easily done by the following standard chemistry formula:

$$C1 \times V1 = C2 \times V2$$

"concentration one $\times$ volume one = concentration two $\times$ volume two".

If a count of 125 million sperm/mL were obtained for the initial suspension, and if 5 mL of standard sperm suspension of 40 million/mL were desired, then the volume of initial suspension to be made up to 5 mL would be calculated as V1:

$$125 \times V1 = 40 \times 5 \quad \text{therefore, } V1 = 1.6 \text{ mL}$$

Determine the density of the mixed suspension of eggs by counting, and adjust to 2000 eggs/mL. Counting can be done by adding to a Sedgwick-Rafter cell, 1 mL or less of the mixed suspension as required, then observing at 20 to 100 $\times$ magnification. It is often useful to dilute an aliquot 10-fold, 100-fold, or, in some instances, 1000-fold, for the purpose of counting. With experience, the original suspension can be diluted according to its appearance, to a few hundred eggs/mL, then a count is made with 0.5 mL. Other techniques of counting

Table 4 Summary of Sperm and Egg Additions to Each Test Vessel for the Three Test Volumes

The numbers of sperm in columns 4/5 and 7 are governed by the sperm:egg ratios of 200:1 and 2500:1 selected as examples.

Initial test volume (mL)	Number of eggs	Volume of egg suspension	Number of sperm (millions) at usual sperm:egg ratios of		Volume of sperm suspension added (mL)	Usual range of concentration in sperm suspension (millions/mL)
			200:1	2500:1		
10	2000	1.0	0.4	5	0.1	4 to 50
5	1000	0.5	0.2	2.5	0.05	4 to 50
2	400	0.2	0.08	1	0.02	4 to 50

¹⁹ Other volumes of sperm solution might be used, with appropriate changes in concentration of the sperm, but larger volumes are not recommended. For example, some existing procedures add 0.5 mL of sperm solution. That would decrease the concentration of the test substance by about 5%, however, and such a change is large enough that it should be allowed for in the calculations of effect. The recommended low volume of 0.1 mL of sperm suspension causes only a small change in concentration that can be neglected for most purposes.

may be used if they are effective. Adjust the suspension to 2000 eggs/mL by adding control/dilution water to reduce the density, or settling eggs and decanting water to increase the density.

For a test with an initial volume of 5 mL, exactly the same procedures are followed except that smaller volumes of the gamete suspensions are added to the test vessels (Table 4). The volume of sperm suspension added would be 0.05 mL (usually containing 2 to 25 million sperm, depending on the sperm:egg ratio required), and the volume of egg suspension added would be 0.5 mL (containing 1000 eggs).

For a test with an initial volume of 2 mL, proportionally smaller volumes of gamete suspensions are used. The added volume of sperm suspension would be 0.02 mL (usually containing 0.8 to 10 million sperm), and the added volume of egg suspension would be 0.2 mL (containing about 400 eggs).

4.2.3 Ratio of Sperm to Eggs

The optimum sperm-to-egg ratio should be determined by trial in each laboratory, as that which gives 90% fertilization under control conditions. Very low fertilization rates in the control would mean that effects of a toxicant on fertilization might be difficult to distinguish from the generally poor and variable background performance. Rates that are too high indicate an excess of sperm that can mask an effect by compensating for part of the toxicity, thus reducing the sensitivity of the test and raising the IC25 and/or NOEC.²⁰ Several options are available for determining a suitable sperm:egg ratio, since the final criterion of a satisfactory test will be the actual rate of fertilization achieved in the control, whether it be the optimum of 90% fertilization, or between the limits of $\geq 50\%$ and $< 100\%$ for a valid test (Subsection 4.5.1).²¹

Ratios that have been reported in the literature to give satisfactory fertilization range from 50:1 to 2500:1 for the various test species (Appendix C). The following sperm-to-egg ratios have been reported to achieve a fertilization range of 70 to 90%:

²⁰ There is some evidence in the literature, of appreciable loss of sensitivity of this test at higher rates of fertilization in the controls, resulting from high sperm:egg ratios. NCASI (1992) found that each of seven paired assays of pulp mill effluent showed decreased sensitivity in the test which had the higher rate of control fertilization (based on IC50s for gametes of eccentric sand dollars). The seven tests that averaged 94% fertilization in controls were 2.2-fold less sensitive, on average, than seven parallel tests with 84% average control fertilization. Another set of eleven assays carried out by NCASI (1992) on a sample of kraft mill effluent, showed a geometric average IC25 of 2.9% effluent at a low sperming ratio, but an IC25 of 6.7% at a high sperming ratio, a 2.3-fold loss of sensitivity. Similarly, Dinnel *et al.* (1987) found that the IC50 of silver for eccentric sand dollars was 23 $\mu\text{g/L}$ at a low sperm-to-egg ratio, but was 37 $\mu\text{g/L}$ at a high sperming ratio, a decrease in sensitivity by a factor of 1.6.

²¹ The target of 90% fertilization success was selected since it helps avoid the problems of too many and too few sperm. It is possible that other agencies might, in the future, adopt standardized tests which stipulate higher fertilization rates under control conditions, perhaps $\geq 95\%$. The objective of those other agencies would be to decrease variability between laboratories, at some sacrifice of sensitivity. Achieving 90% fertilization often requires a fairly delicate balance of the gamete ratio and other factors, and suitable procedures must be established within each laboratory.

green sea urchin, 2000:1; Pacific purple sea urchin, commonly 200:1 but up to 2000:1; eccentric sand dollar, often about 1200:1 but also reliably reported in the range 50:1 to 200:1; and *Arbacia*, 2500:1. Such general guidance cannot, however, be depended on to yield satisfactory test results in any given laboratory or season. Canadian interlaboratory tests, for example, found that some sperm:egg ratios had to be an order of magnitude higher than values mentioned above (Miller *et al.*, 1992).

Ideally, the appropriate sperm:egg ratio should be determined just before each test, and with the gametes to be used in that test. The pre-test trial might be shortened and simplified to use one or two sperm:egg ratios that are thought to be low. Results could be used to position the gametes that are to be used on a "curve of fertilization success" from past experience in the laboratory, allowing an appropriate ratio to be selected for the real test.

In practice, experience at a given laboratory can establish a "standard" ratio that usually gives the desired results for a particular species. However, the routine use of a "standard" ratio risks lowering the quality of testing. If the standard ratio yielded less than 50% fertilization in the control, or 100% fertilization (Subsection 4.5.1), the test would be invalid and would have to be repeated using a different ratio. Other tests might lose sensitivity because of "over-sperming". The sperm:egg ratio might require adjustments with season, and 10-fold changes in requirements due to season are not unknown (personal communication, G.A. Chapman, U.S. EPA, Newport, Oregon).

Because of the normal variation in percent fertilization for controls, a pre-test trial of

this is highly recommended ("control pre-trial"). Investigators familiar with the echinoid fertilization assay find that the time spent in a control pre-trial for each test has, in the long run, saved considerable time, money, and sometimes irreplaceable samples. For example, one laboratory routinely runs control pre-trials "on a matrix of gametes from at least two females and two males with at least three different sperm dilutions per male" (Carr and Chapman, 1992).

An alternative approach to circumvent control pre-trials is to include replicates of two or three sperm:egg ratios for each concentration used in the test including controls. Results for the ratio that yielded a fertilization rate closest to 90% in the control would be used in calculating the NOEC/LOEC, or ICp, or both (Section 4.5). NCASI (1992) points out that this actually requires less of the investigator's time than running a control pre-trial and then a test, and has a further advantage of avoiding any changes of sperm activity during the interval from pre-test to test.

If the sperm:egg ratio was determined by a control pre-trial, or arbitrarily selected, the strength of the suspension of sperm is also fixed (Subsection 4.2.2). For example, if a sperm:egg ratio of 2000:1 were required for the 2000 eggs to be added, then 4 million sperm would be needed in the 0.1 mL of added suspension, or 40 million sperm per mL in the suspension.

4.2.4 Exposure of Gametes

Individual vessels are positioned for the exposure in a test tube rack or other rack, held in the water bath or other temperature-control facility. Vessel positions in the rack must be either completely randomized, or randomized in "columns" of

the rack, each column representing one replicate of each concentration and control.²² Each vessel must be clearly labelled or positions coded so that concentrations and replicates can be identified.

Temperature, salinity, dissolved oxygen, and pH levels in aliquots of the test solutions should be checked and adjusted, if required/permitted, to acceptable levels (Section 4.3) before adding the solutions to the test vessels.

The test has three options for duration of exposure, options which are otherwise identical in their procedures. Obviously, only one of these options can be used in a given test, and for comparative tests. The shortest option is the standard exposure for normal testing and monitoring. It is 10 min of sperm exposure, with the addition of eggs at that time and an exposure that continues for a further 10 min of sperm plus eggs, i.e., the 20-min test.

Either of two longer exposures might be used for special purposes such as research or comparison with other data. The second option is 20 min of sperm exposure followed by 20 min of sperm plus eggs, the 40-min test. The longest option is exposure of the sperm for 60 min, plus 20 min, an 80-min test.²³

The three options for volume of test solution are independent of the options for duration (thus nine options for test procedure). The option for an initial test volume of 10 mL is the usual standard and is described here.²⁴ The procedures for the smaller test volumes of 5 and 2 mL would be identical except that proportionally smaller volumes of gamete suspensions would be added (Table 4).

The solution of sperm is mixed, and to start the test, 0.1 mL is added to each test vessel, which already contains 10.0 mL of test solution (Subsection 4.1.2). At the end of the sperm exposure, the egg preparation is mixed and 1.0 mL is added to each test

²² The randomized locations may be selected in various ways, and most laboratories will have customary procedures. Some laboratories have computer programs tailored to the needs of the echinoid test, which make use of the random numbers available in standard computer software. For a guarantee of objectivity and assured validity of statistical tests, a "blind" test could be done by having all vessels including controls and reference toxicants, coded by a person other than the one who will make the count of fertilization.

²³ The longer exposure is usually acknowledged as a more sensitive test of toxicity. The improved detection of a toxic effect is less pronounced, however, because there is usually a drop in fertilization rate in the control, as the sperm are held for longer periods before the addition of eggs. The drop in control fertilization might be as great as from 90% for a 10-min exposure of sperm to 30% for a 60-min exposure (Pagano *et al.*, 1983). The apparent margin between performance of the control and test concentrations might not improve greatly with the longer exposure of sperm (test of cadmium, Pagano *et al.*, 1986). Investigators in British Columbia have noted such a decreased rate of control fertilization in eccentric sand dollars, comparing sperm exposures of 10 minutes and 30 minutes (personal communication, G.C. van Aggelen, B.C. Ministry of Environment).

The short exposure (10 + 10 min) was favoured as a standard test by Canadian investigators participating in an interlaboratory comparison (Miller *et al.*, 1992). The short exposure might partly compensate for loss of sensitivity by reduced variability. For example, in seven paired tests of toxicity of bleached pulp mill effluent to eccentric sand dollars, the short test (20 min total) was less sensitive by a factor of 2.4 compared to the long test (80 min total), but had a smaller coefficient of variation by a factor of 2.0 (NCASI, 1992).

²⁴ The 10-mL volume was favoured by Canadian investigators in an interlaboratory comparison, and was adopted as the standard method in the trials (Miller *et al.*, 1992). There was concern that the smaller volumes might decrease the precision in handling small volumes of fluid, and might increase the "edge effects" at surfaces contacting the air and test vessels.

vessel. Automatic dispensing micro-pipettes are needed to accomplish these steps within narrow time limits. Care must be taken when adding sperm and eggs to the vessels; all of the fluid delivered from a pipette must enter the test solution rather than striking the side of the vessel, and the pipette tip must not touch the test solution. The suspension of gametes should be mixed after every second or third vessel is filled. After sperm have been added to all vessels, and again after eggs have been added, all vessels should be thoroughly mixed by swirling, in-and-out pipetting, or brief use of a vortex mixer.

A timing procedure should be used for adding sperm to vessels in sequence, for example one vessel every 5 seconds. The eggs should be added to the vessels in the same sequence (order of vessels) and with the same timing interval as was used for sperm, in order to equalize exposure periods. Termination of the test should again be done in the same sequence with the same timing. Additions to test vessels should not be done according to magnitude of concentration, but by replicate, i.e., the first set of replicates, then the second, then the third (Chapman, 1992).

At the end of the sperm-plus-eggs exposure, the test is terminated by adding either 2 mL of 1% glutaraldehyde, or 2 mL of 10% buffered formalin to each test vessel.²⁵ (The amounts of preservative are divided by 2 and by 5 for the two smaller-volume test options.) Preserved eggs should be counted

within three days of test completion. During storage, vessels containing eggs should be sealed (e.g., using plastic film).

4.3 Test Conditions

This is a static test without renewal of test solutions. The test is carried out at 15°C for the four native species, and 20°C for the listed non-native species. Salinities in all test vessels are normally within 1 g/kg of the control, in the range 28 to 34 g/kg. An attempt is made, if necessary, to raise the dissolved oxygen of all test solutions above 40% saturation.

4.3.1 Temperature

A test temperature of 15°C should be used for green sea urchins, Pacific purple sea urchins, and eccentric sand dollars. The test temperature should be 20°C for the non-native *Arbacia* and white sea urchins. Temperatures of all test solutions should be within 1°C of the intended value as determined by measurements in aliquots or test vessels without gametes (dedicated to temperature monitoring). Temperatures should be measured at the beginning of the test, and at representative times during the test.

The test temperatures recommended here are 3° to 6°C higher than the values recommended for holding the adults of the same species, but within the bio/kinetic ranges. These somewhat elevated temperatures should make the test more

²⁵ Formalin is a 37% to 40% solution of formaldehyde. To make 10% buffered formalin, add 100 mL of formalin, 4 g monobasic sodium phosphate, and 6.5 g anhydrous dibasic sodium phosphate to 900 mL of distilled water. Formalin is a hazardous substance, must be handled in a fume hood, and counting of eggs must take place with similar positive ventilation. Work with glutaraldehyde should also take place in a fume hood or well-ventilated area. Both substances should be kept away from areas used to hold and test organisms. Some authors add chlorine bleach solution immediately before enumeration of eggs, to neutralize excess formalin.

sensitive in detecting some toxicants.²⁶ Some of the recommended temperatures conform with those previously used in Canadian methods or U.S. standard methods, but they necessarily diverge from some other methods, because of the variety employed elsewhere (Appendix C).

4.3.2 Salinity

A standard test should be carried out at a salinity of 30 g/kg. All test solutions should be in the 28 to 34 g/kg range, and they should also be within 1 g/kg of the salinity of the control.²⁷

If a chemical is being tested, it should be made up to the test concentrations using a control/dilution water (Subsection 4.1.1) which has a salinity in the required range. Salinity of aqueous samples (e.g., effluents) should be measured before the test, and if necessary, should be adjusted using brine, sea salts, or deionized or uncontaminated fresh water, as appropriate (see Subsection 2.3.4).

Samples of effluent, leachate, receiving water, and elutriate or other aqueous extract

from sediment could also be tested without adjusting salinity of the sample, if it were desired to assess the total effect, including divergent salinity. It should be realized that if the sample is essentially fresh water (salinity <5 g/kg) or is a brine, the results of the toxicity test will probably reflect unfavourable salinity rather than any toxic substance(s) in the sample. If an unadjusted sample were tested, it would be desirable to run a set of salinity controls using parallel concentrations of distilled water (Subsection 4.1.4), or to conduct a second test with salinity of the sample adjusted, or both, in order to understand the contribution of salinity to toxicity.

4.3.3 Dissolved Oxygen and Aeration

If (and only if) calculations from the dissolved oxygen measured in the sample to be tested indicate that one or more of the test concentrations would be outside the 40 to 100% range of air saturation, the sample or an aliquot of sample should be aerated before starting the test ("pre-aeration"). Oil-free compressed air should be dispensed through a disposable glass pipette, with bubble size 1 to 3 mm, at a minimal rate for

²⁶ In fertilization assays at a favourable salinity of 28 g/kg, the IC50s for silver decreased appreciably over the temperature series 7°, 12°, and 17°C. For green sea urchins the IC50s were respectively 215, 110, and 38 µg/L, while for eccentric sand dollars a smaller change of IC50s was 120, 88, and 66 µg/L (Dinnel *et al.*, 1982). The IC50s for the insecticide endosulfan did not appear to be affected in a predictable way by test temperature.

²⁷ Gametes and larvae of echinoids are considered to have a fairly narrow range of salinity tolerance, and low salinity can have major effects on the estimate of toxicity in a fertilization assay. For instance, the fertilization rate for the Pacific purple sea urchin decreased at a salinity of 28 g/kg compared to 30 g/kg, with no toxicant present (Oshida *et al.*, 1981). Contrasting work showed no decrease in fertilization rate of Pacific purple sea urchins until salinity was below 20 g/kg (Dinnel *et al.*, 1987), a finding that might be partly explained by a high ratio of sperm to eggs, since that can partly compensate for low salinity. Green sea urchins showed a decreased rate of fertilization at 24 g/kg and less (Dinnel *et al.*, 1987). Increased sensitivity to toxicants can be caused by lowered salinity (ASTM, 1990). For example, green urchins tested at 10°C showed IC50s for silver of 94 µg/L, 45 µg/L, and 34 µg/L at salinities of 30, 28, and 26 g/kg respectively, although IC50s for the insecticide endosulfan showed little or no change (Dinnel *et al.*, 1987). Lowered salinity was particularly effective in causing increased toxicity of silver, with green sea urchin fertilization, when the test was done at high temperature (17°C) or low temperature (7°C), while there was little salinity effect at a near-optimal temperature of 12°C (Dinnel *et al.*, 1982). Similar effects of decreased salinity were shown with a South African sea urchin (genus *Parechinus*) for which fertilization success dropped off steadily as salinity decreased from that of normal seawater. The eggs had an optimal salinity in the range 28 to 37 g/kg, and were more sensitive than sperm (Greenwood and Bennett, 1981).

effective aeration of the particular vessel and volume of fluid being used. Duration of pre-aeration should be the lesser of 20 minutes and attaining 40% saturation (or 100% saturation, if supersaturation is evident).²⁸ Any pre-aeration should be discontinued at 20 minutes and the test initiated, whether or not 40 to 100% saturation was achieved in the aliquot of sample, or would be expected in all test solutions. Dissolved oxygen must then be recorded for the start of the test in representative aliquots of the test solutions including the highest concentration. Any pre-aeration must be reported (Section 9).

If oxygen in one or more test vessels is below 40% of saturation, the test becomes invalid as an assessment of the toxic quality, *per se*, of the substance being tested. The test would still be a valid assessment of the total effect of the substance (e.g., effluent) including its deoxygenating influence.²⁹ The required use of oxygen-saturated control/dilution water will, in most instances, result in dissolved oxygen levels

that should not have a large influence on test results.

4.3.4 pH

The pH must be measured in aliquots of the control(s), high, medium, and low concentrations before beginning the test.

Toxicity tests for regulatory or monitoring purposes would normally be carried out without adjustment of pH.³⁰ However, if the sample of test substance causes the pH of any test solution to be outside the 7.5 to 8.5 range, results might reflect effects due to pH alone.³¹ If it is desired to assess toxic chemicals *per se* rather than the deleterious or modifying effects of pH, then the pH of the solutions or sample should be adjusted, or a second, pH-adjusted test should be conducted concurrently. For an adjusted test, the initial pH of the sample, or of each test solution may, depending on objectives, be adjusted to within ± 0.5 pH units of that of the control/dilution water, before exposure of the gametes. Another acceptable approach for an adjusted test is to change each test solution, including the control,

²⁸ Aeration can strip volatile chemicals from solution, or increase their rate of oxidation and degradation to other substances. However, aeration of a sample before exposure of gametes might be necessary due to the oxygen demand of the test substance (e.g., oxygen depleted in the sample during storage). Because of the small volumes of test solutions for the fertilization assay, aeration of individual concentrations is not practical, and aeration of an aliquot of sample is carried out if necessary.

²⁹ The lower limit of 40% saturation for dissolved oxygen in test solutions is an arbitrary value, because oxygen levels well above that are stressful to most aquatic organisms and probably affect gametes also. Stress from low oxygen might interact with any stress from toxicants, and be measured as part of the effect of the sample, be it effluent or other test substance. Any such interaction at DO >40% saturation has been accepted in this test procedure, as part of the impact being measured.

³⁰ A justification for not changing the pH of the sample or solution is that pH can have a strong influence on the toxicity of a substance being tested. Thus, any change caused in the pH of the receiving water, with concomitant modification of toxicity, could be accepted as part of the pollution "package". That leads to the rationale that the pH should not be adjusted in tests.

³¹ Reproduction in the green sea urchin is known to be sensitive to pH (Starr, 1990). Although a European sea urchin showed that sperm viability was prolonged when pH ranged from 6 to 7.5, compared to about pH 8, there was nevertheless clearly evident damage to the sperm below pH 8, since subsequent developmental abnormalities increased at pH 7.5 and mitotic abnormalities increased at pH 7 (Pagano *et al.*, 1985).

upwards to pH 7.5 to 8.0 (if the solution has pH <7.5), or downwards to pH 8.0 to 8.5 (if the solution has pH >8.5). Solutions of hydrochloric acid (HCl) or sodium hydroxide (NaOH) at strengths $\leq 1 N$ should normally be used for all pH adjustments. Some situations (e.g., effluent samples with highly-buffered pH) might require higher strengths of acid or base.³²

In some circumstances it might be desired to carry out the most sensitive test possible for detecting toxic chemicals, rather than including pH as part of the total effect of a chemical, effluent, leachate, or elutriate. In such a case, any effect of low or high pH, in changing viability of gametes and success of fertilization, should be eliminated by adjusting pH of test solutions as necessary, to the preferred range of 8.0 to 8.2.³³

Abernethy and Westlake (1989) provide useful guidelines for adjusting pH. Aliquots of samples or test solutions receiving pH-adjustment³⁴ should be allowed to equilibrate after each incremental addition of acid or base. The amount of time required for equilibration will depend on the buffering capacity of the solution/sample.

For effluent samples, a period of 30 to 60 min is recommended for pH adjustment (Abernethy and Westlake, 1989). For an echinoid test, the adjustment would be made on aliquots used to prepare test concentrations, the pH in each would be recorded (Section 4.4), and the test started with no further attempt at adjustment.

If the purpose of the toxicity test is to gain an understanding of the nature of the toxicants in the test substance, pH adjustment is frequently used as one of a number of techniques (e.g., oxidation, filtration, air stripping, addition of chelating agent) for characterizing and identifying sample toxicity. These "Toxicity Identification Evaluation" (TIE) techniques provide the investigator with useful methods for assessing the physical/chemical nature of the toxicant(s) and their susceptibility to detoxification (U.S. EPA, 1991a; 1991b).

4.4 Test Observations and Measurements

At the end of the exposure, preserved eggs are taken from each test vessel after mixing³⁵, and an equal number from each

³² The rationale for making these adjustments is not really contradictory to the previous rationale of not adjusting pH of wastewaters, but depends on the purpose of the test. Some chemicals and wastewaters will create levels of pH that have direct sublethal or lethal effects, especially in monitoring or compliance tests with full-strength effluent. An investigator might not be primarily interested in whether extreme pH is toxic, because such a pH would be unlikely after even moderate dilution in receiving seawater, which is naturally well-buffered. If pH *per se* were of primary interest, it could be economically assessed by physicochemical measurements. An investigator would often wish to know if toxic substances were present in a wastewater, and detecting them would require elimination of any masking by toxic action of pH. That rationale leads to the use of pH-adjusted samples or test solutions, in a parallel manner to the standardization of temperature, salinity, and dissolved oxygen at favourable levels when testing for toxic substances.

³³ Of the six existing procedural documents which indicate pH requirements for the fertilization assay, five of them specify values in the range 7.8 to 8.2 (Appendix C).

³⁴ Tests with a chemical, effluent, leachate, elutriate, or aqueous extract of a sediment, which are to receive pH adjustment, might require the separate adjustment of each test solution including the control. Tests with receiving water would normally adjust an aliquot of the undiluted sample, before preparing the test concentrations.

vessel, in the range of 100 to 200 eggs, is counted and classified as either fertilized or not fertilized (Figure 3). The count is made under a microscope at 100× magnification, preferably by phase-contrast microscopy. A counting cell such as a Sedgwick-Rafter chamber might be useful, although the count can be made using an etched petri plate. Microscopic technique is important, and can affect the accuracy of the counts. Consistency of counting should therefore be checked by trials, especially among different people who might be involved in counting.

The criterion of fertilization is a raised fertilization membrane, and this includes full, partial or collapsed membranes (see Figure 3), none of which are seen in unfertilized eggs (NCASI, 1991).³⁶

Artifacts such as partial collapse of membrane or movement of the egg to one side of the hyaline sphere, can occur during preservation after the test. Clearly abnormal eggs, or dead ones, are simply omitted from the count, whether they are fertilized or not. The counts are recorded for each test vessel.

4.5 Test Endpoints and Calculations

The biological endpoint of the test is adverse effect on success of fertilization, assessed by

comparison with the controls. Percent fertilization is calculated for each test vessel.

Various statistical endpoints can be calculated from percent fertilization, and the rationale and methods of calculation follow and are discussed in detail in U.S. EPA (1988). The *NOEC* and *LOEC* can be derived statistically by the hypothesis-testing approach, and this is recommended as a primary technique. Calculation of the *inhibiting concentration for a specified percent effect (ICp)* is also recommended. Advice should be sought from a statistician in carrying out the analyses of results.

4.5.1 Validity of Test

The test is invalid if fertilization rate in the control water is less than 50%, or if complete fertilization is achieved, i.e., 100% fertilization in all replicates of the control.³⁷ A positive and logical dose-effect curve must have been attained, for the results to be considered valid, i.e., the effect on fertilization must become generally greater at higher concentrations. Additionally, the required physical and chemical conditions must have been met.

If dissolved oxygen in one or more test vessels was less than 40% saturation, the test should be considered an invalid assessment of the toxic quality, *per se*, of the substance

³⁵ Most of the eggs are normally in the lower part of the test vessel and could be sampled from there. However, unfertilized eggs are sometimes adhesive and might clump together on the glass. This could bias the results, whether the clumps happen to be over-sampled or under-sampled. A remedy is to pipette off much of the overlying test solution, in order to concentrate the suspension of eggs, then mix and sample for counting.

³⁶ High or low pH, or high temperature, can cause false fertilization membranes, and if that were of concern for a particular test sample, a separate vessel or vessels at high concentration could be carried through without preservation, and checked the following day for normal development into larval stages. A set of unfertilized "blanks" (i.e., test vessels with unfertilized eggs added to the test solutions but containing no sperm) might also be included in the test in order to identify false fertilization membranes.

³⁷ Although 90% fertilization is aimed for in the test, and that would be a satisfactory result for the control, the lower limit for validity has been set at ≥50% control fertilization, since useful information might still be obtained from such a test. The upper limit of validity has been set at *less than 100%*, since high fertilization rates are indicative of over-sperming and are associated with loss of test sensitivity.

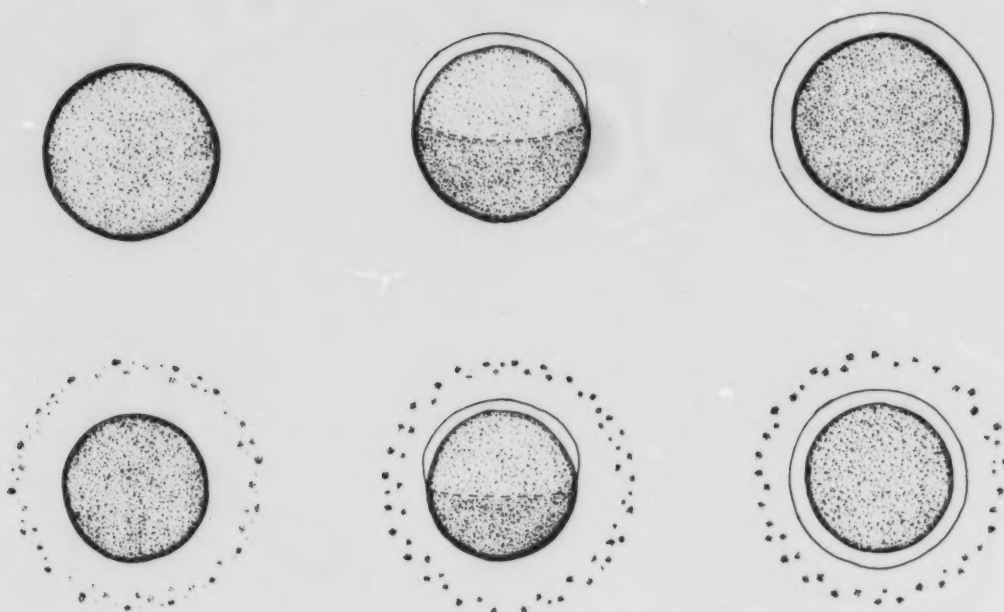


Figure 3

Discriminating Between Fertilized and Unfertilized Eggs.

Outlines of eggs as seen under a dissecting microscope. The three drawings in the upper row represent a sea urchin such as the green sea urchin. The egg on the left is not fertilized. The middle egg has a fertilization membrane that is partially raised and is considered fertilized. The right-hand egg has a completely raised fertilization membrane. The Pacific purple sea urchin is similar but within the outer fertilization membrane, an inner hyaline membrane might be evident. The three drawings in the lower row represent the eccentric sand dollar, from left to right, unfertilized, fertilized with a partially raised membrane, and with a completely raised membrane. The jelly-like coating of the sand dollar contains pigment granules and usually disappears during later development of the egg. Drawn by M.A. White, from prepared slides from McGibbon and Moldan (1986), and from drawings of Kelley Battan of NCASI, Anacortes, Washington.

being tested. The test would still be a valid assessment of the total effect of the test substance (Subsection 4.3.3).

4.5.2 No-Observed-Effect Concentration

The NOEC and LOEC are determined from the values for percent fertilization in each replicate of the control and the various concentrations. If there is zero fertilization in all the replicates of a given concentration, that concentration is excluded from calculations to determine the NOEC/LOEC.

The statistical procedures to be followed are given in TOXSTAT³⁸ (Gulley *et al.*, 1989). An up-to-date version of TOXSTAT is available on computer disk (see Appendix B). The methods in TOXSTAT start with a check of normality and homogeneity of variance, and provide suitable tests of significance for particular types of distribution.

If the data are regular or can be made so by suitable transformation, an analysis of variance is carried out. Differences of each concentration from the control can be assessed by Dunnett's test, a standard multiple-comparison test. Dunnett's test provides estimates of the Minimum Significant Difference (MSD), which is the magnitude of the difference in average success of fertilization, that would have to exist between the control and a test concentration, before a significant effect could be concluded for that concentration. Dunnett's test is not a particularly powerful way of discriminating effects in toxicity tests since it ignores the information on the ordering of the test concentrations by magnitude (Masters *et al.*, 1991). Williams'

test is also available in TOXSTAT and is designed to be sensitive to the association between the degree of effect and the ordering of concentrations by magnitude (Gulley *et al.*, 1989). Williams' test (Williams, 1971; 1972) is recommended as an alternative to Dunnett's test. If there are unequal numbers of replicates because of accidental loss or other cause, the *Bonferroni t-test* is substituted for Dunnett's or Williams' test.

If a set of data cannot meet the requirements for normality or homogeneity, and cannot be transformed to do so, there are nonparametric tests provided in TOXSTAT which may be substituted (*Steel's many-one rank test*, or the *Wilcoxon rank sum test* in the case of unequal replicates). Those nonparametric options may be used, and are powerful tools for data that are not normally distributed. The nonparametric tests are less powerful than parametric tests, however, when used on normally-distributed data, and in that situation they might fail to detect real differences in effect, i.e., an underestimate of toxicity might result. It should also be remembered that four replicates are required to make use of the nonparametric methods.

A geometric average of the NOEC and LOEC is often calculated for the convenience of having one number rather than two. Such a value may be used and reported, recognizing that it represents an arbitrary estimate of an effect-threshold that might lie anywhere in the range between the LOEC and NOEC. The calculated value of the geometric mean is governed by whatever concentrations the investigator happened to select for the test. No confidence limits can be estimated statistically for the NOEC and

³⁸ The methods of TOXSTAT are not detailed here because the instructions are best followed in the written description that accompanies the programs on computer disk. Briefly, data are tested for normality by the *Shapiro-Wilks* test, and for homogeneity by *Bartlett's test*. If the data do not meet the requirements, it might be possible to transform them with logarithms or arc-sine to meet the requirements. The transformation can reduce the sensitivity of the analysis and the ability of the toxicity test to detect differences.

LOEC, and that is also the case for their geometric mean, although the NOEC and LOEC indicate the outer limits of the estimate. The geometric mean of NOEC and LOEC is often called the *chronic value* in the United States, or sometimes the *subchronic value*, but those terms are misleading for this test with gametes which represents an extremely small fraction of an echinoid's lifetime and therefore does not approach a chronic exposure. A recommended term for the geometric average of NOEC and LOEC is *TEC* signifying *threshold-effect concentration*. The use of "threshold" is in the dictionary sense of "point at which an effect begins to be produced".

4.5.3 Inhibiting Concentration

As an alternative or additional endpoint which is becoming widely used, the ICp (inhibiting concentration for a specified percent effect) may be calculated as a point-estimate of the concentration causing a certain degree of effect. The percentage is selected by the investigator, and is customarily 25% or 20% reduction in performance rate compared to the control.³⁹ In this test, the IC25 is recommended and would be the concentration estimated to cause a 25% reduction in success of fertilization compared to the control.

The ICp is often a more useful and desirable measure of effect than estimates of NOEC/LOEC because confidence limits can be calculated, allowing statistical comparisons with ICps in other tests

(Suter *et al.*, 1987). Such comparisons cannot be approached in as logical a manner, or with tests for significant differences, by means of the hypothesis-testing approach used for NOEC/LOEC.

The observation of percent fertilized eggs at each concentration is used to calculate the inhibition of fertilization, as a percent reduction compared to the control. The *Percent Reduction in Fertilization* for a given concentration is calculated as:

$$PRF = \frac{CF - OF}{CF}$$

Where:

PRF = Percent Reduction in Fertilization;

OF = Observed percent Fertilized eggs in a given test vessel; and

CF = Control percent Fertilized eggs in dilution/control water.⁴⁰

An analysis of percent reduction in fertilization, to determine an ICp, should begin with a hand plot of PRF values against the logarithm of test concentration. The IC25 (or other ICp) would be read from an eye-fitted line. This graphic estimate would serve to check whether results from mathematical computations were reasonable. The graph would also show whether a positive and logical relationship was obtained between concentration and effect,

³⁹ Some work with other sublethal tests has indicated that the IC25 or IC20 is a useful measure of sublethal effect that is similar in magnitude to the LOEC.

⁴⁰ This formula gives the same result as Abbott's formula applied to the proportion of *unfertilized* eggs. Abbott's formula is a correction for control performance that is commonly used in aquatic toxicology (Appendix C, part 13). The formula shown here gives the same numerical result as a calculation of reduced fertilization based on the corrected values derived from the formula of ASTM (1990), shown in Appendix C, part 13. Calculation by the formula shown here provides percent reductions that can be directly plotted, or used in calculations to estimate an ICp.

one of the requirements for validity of the test (Subsection 4.5.1).

A mathematical estimate of the ICp can be obtained by a straightforward *linear interpolation* method (U.S. EPA, 1989, Appendix J). Confidence limits about the ICp can be estimated by a "bootstrap" method on computer (BOOTSTRP), which incorporates linear interpolation to estimate the ICp itself (Norberg-King, 1988; U.S. EPA, 1989). The BOOTSTRP program can be obtained on a user-supplied disk from an Environment Canada regional office (Appendix B).⁴¹ Use of this program requires a computer with a math co-processor. Probit analysis to estimate an effective concentration (EC50 or EC25) is not valid for reasons given under ICp in the Terminology section.

Some common-sense limitations must be applied to estimates of ICp. It should not be estimated on the basis of an extrapolation, i.e., the data should extend above and below the percent effect of interest. For example, if it were desired to estimate the IC25, there should be at least one concentration causing greater than 25% effect (but less than 100% effect), and at least one causing less than 25% effect (but greater than 0% effect). If, for some reason, it is desired to estimate an extreme value such as IC05 or IC95, the

foregoing restriction may be relaxed, but there must be at least one concentration causing a partial effect near the ICp of interest, say an effect that did not differ by >5% from the one of interest.⁴² Variability is great near the extremes of the relationship, and in particular, observed effects of 0% and 100% often add little information to the estimate of ICp.

4.5.4 Other Test Designs and Purposes

In a single-concentration test, a t-test is normally the appropriate method of comparing the data from the test concentration with those of the control, and the procedure for a t-test can be taken from any statistics textbook. If percent fertilization in the test concentration is significantly lower than the same statistic for the control, it is accepted that the test substance is having an effect.

Requirements for homogeneity of variance and normality must be satisfied (Appendix H of U.S. EPA, 1989; Gulley *et al.*, 1989) before using the standard t-test. If the data do not satisfy the requirements, a nonparametric test could be selected with advice from a statistician; no particular test appears to have become standard practice as yet.

⁴¹ At present, linear interpolation using BOOTSTRP appears to be the only method that is in common use and easily available, for obtaining confidence limits on an ICp. Investigators should be alert for improved methods which might become available, however, since there are some undesirable features of linear interpolation, with or without BOOTSTRP. First, there is a requirement that "the responses are monotonically non-increasing" (U.S. EPA, 1989), i.e. in the echinoid assay, a better fertilization rate should not prevail at a high concentration than at a lower concentration. That is not always the case in toxicity assays, and the correction procedure in linear interpolation can bias the estimate of ICp. Second, the ICp is interpolated between two bracketing concentrations, and the rest of the relationship between concentration and effect is not used, as it would be, say, in using probit analysis to estimate a lethal concentration by fitting a line to the entire distribution of data. Third, the interpolation to estimate the ICp is done on an arithmetic basis of concentration instead of a logarithmic one, which would also affect the value derived.

⁴² The quality and distribution of other data in the test also influence the value of the estimate of an extreme ICp and no firm guideline can be given for the required closeness of an observed data-point to the effect of interest. The spread of the confidence limits will always indicate the reliability of the ICp.

In some cases, the test will not involve various concentrations of a single sample of test substance, but rather a set of different samples, such as full-strength effluents from different industries, or samples of seawater from different places. It might be desired to test whether each sample is different from the control, and that could be done using one option in the statistical program TOXSTAT, with Dunnett's test as the appropriate one for comparison with the control. It might also be an objective, to determine whether the samples were different from each other, and the appropriate statistical test in that case would be Tukey's test. Such sets of tests cannot report ICp or NOEC/LOEC for each sample tested, but should report the observed percent fertilized eggs, and whether that number was significantly different from the control(s).

4.6 Reference Toxicant

The routine use of a reference toxicant or toxicants is necessary to assess the relative sensitivity of the batches of gametes that are used, under standardized test conditions, and the precision and reliability of data produced by the laboratory for the selected reference toxicant(s) (Environment Canada, 1990d).

Sensitivity of gametes to the reference toxicant(s) must be evaluated at least once each calendar month in which the fertilization assay is performed; assessments every two weeks would be more satisfactory and are recommended. The test using the

reference toxicant is most useful when carried out simultaneously with an actual toxicity test, and ideally, one would be carried out for each batch of gametes used in toxicity tests.

Criteria considered in recommending appropriate reference toxicants for this test include:

- chemical readily available in pure form;
- stable (long) shelf life of chemical;
- highly soluble in water;
- stable in aqueous solution;
- minimal hazard posed to user;
- easily analyzed with precision;
- good dose-response curve for echinoid gametes;
- known influence of pH on toxicity of the chemical, in this test; and
- known influence of salinity on toxicity of the chemical, in this test.

Copper is recommended for use as the reference toxicant for this test.⁴³ Gamete sensitivity should be evaluated by tests following the standard methods and conditions given in this document, to determine the ICp or NOEC/LOEC for

copper. Copper sulphate or copper chloride should be used for preparing stock solutions, which should be acidic (pH 3 to 4), and may be used when prepared, or stored in the dark at $4 \pm 2^\circ\text{C}$ for several weeks before use. Concentration of copper should be expressed as $\text{mg Cu}^{++}/\text{L}$.

Natural or reconstituted seawater is to be used for controls and dilution. To provide a high degree of standardization for the reference toxicant tests, the salinity of the control/dilution water should be adjusted to a consistent value that is favourable to the gametes, in the range 28 to 34 g/kg, preferably 30 g/kg.

Concentrations of reference toxicant in all stock solutions should be measured chemically by appropriate methods (e.g., APHA *et al.*, 1989). Upon preparation of the test solutions, aliquots should be taken from at least the control, low, middle, and high concentrations, and analyzed directly or stored for future analysis should the ICp or NOEC be atypical (outside warning limits). If stored, sample aliquots must be held in the

dark at $4 \pm 2^\circ\text{C}$. Copper solutions should be preserved before storage (APHA *et al.*, 1989). Stored aliquots requiring chemical measurement should be analyzed promptly upon completion of the toxicity test. Calculations of ICp or NOEC should be based on measured concentrations if they are appreciably (i.e., $\geq 20\%$) different from nominal ones and if the accuracy of the chemical analyses is satisfactory.

A warning chart (Environment Canada, 1990d) should be prepared and updated for each reference toxicant used. Successive ICps or NOECs are plotted on this chart and examined to determine whether the results are within ± 2 SD of values obtained in previous tests. The geometric mean ICp or NOEC together with its upper and lower warning limits (± 2 SD calculated on a geometric [logarithmic] basis)⁴⁴ are recalculated with each successive ICp or NOEC until the statistics stabilize (U.S. EPA, 1989; Environment Canada, 1990d).

If a particular ICp or NOEC falls outside the warning limits, the sensitivity of the gametes

⁴³ No clear choice of a standard reference toxicant seems to have been made by the various groups carrying out echinoid fertilization assays, and most do not specify a reference toxicant or deal with the subject (Appendix C). Copper and sodium azide have both been used by two groups in the United States and have been selected for use in B.C. provincial laboratories, along with silver (personal communication, G.C. van Aggelen, B.C. Ministry of Environment). For copper, IC50s in the vicinity of 20 to 26 $\mu\text{g Cu}/\text{L}$ and coefficients of variation from 23% to 46% have been reported (personal communication, G.A. Chapman, U.S. EPA; U.S. EPA, 1988). Less information is available for sodium azide and it is not mentioned in the guide to reference toxicants (Environment Canada, 1990d). Stock solutions of sodium azide can be stored for a maximum of three months.

Other chemicals might be tried as potential reference toxicants. Cadmium has been found by IGATG (1991) to have a threshold-effect concentration (TEC) of 18 $\mu\text{g Cd}/\text{L}$ for green and white sea urchins. Cadmium is not, perhaps, the most desirable chemical for standard use since it is a dangerous bioaccumulative toxicant. Cadmium has also been reported to be associated with a high proportion of deformed eggs which are difficult to classify when counting (personal communication, G.C. van Aggelen). Reagent-grade phenol might be useful since it would be an organic chemical to complement the inorganic copper, and since phenol is already recommended for other standard toxicity tests of Environment Canada (1990a; 1990b; 1992b). Phenol stock solutions should be made up on the day of use. Sodium dodecyl sulphate has also been used with fertilization assays, but has some undesirable features as a reference toxicant (Environment Canada, 1990d).

⁴⁴ If the ICps or NOECs fail to show a lognormal distribution, an arithmetic mean and SD might prove more suitable. Use of the ICp is recommended, in preference to the NOEC or even the TEC, since it is a point estimate and less influenced by the particular concentrations and dilution factor selected for the test.

and the test system are suspect. Inasmuch as this can occur 5% of the time due to chance alone, an outlying value does not necessarily mean that the sensitivity of the batch of gametes or the precision of the toxicity data produced by the laboratory are in question. Rather, it provides a warning that this might be the case. A check of all holding and test conditions is required at this time.

One check that might be made in such circumstances is the fertilization success for various sperm:egg ratios, compared with the range of values previously obtained. That assessment should provide a useful indication of decreasing viability of gametes, as might occur, perhaps, at the end of a spawning season.

Test endpoints that usually fall within warning limits do not necessarily indicate that a laboratory is generating consistent results. A laboratory that produced extremely variable data for a reference toxicant would have wide warning limits; a

new data-point could be within the warning limits but still represent undesirable variation in results. A coefficient of variation of 20% or 30% is tentatively suggested as a limit by Environment Canada (1990d). That seems a reasonable range but the matter has not been studied for the echinoid fertilization assay.

4.7 Legal Considerations

Care must be taken to ensure that samples collected and tested with a view to prosecution will be admissible in court. For this purpose, legal samples must be: representative of the substance being sampled; uncontaminated by foreign substances; identifiable as to date, time, and location of origin; clearly documented as to the chain of custody; and analyzed as soon as possible after collection. Persons responsible for conducting the test and reporting the findings must maintain continuity of evidence for court proceedings (McCaffrey, 1979), and ensure the integrity of the test results.

Section 5

Specific Procedures for Testing Chemicals

This section gives particular instructions for testing chemicals, in addition to the procedures in Section 4. For chemicals, a multi-concentration test is usually performed to determine the IC_p and/or NOEC/LOEC. Three replicates, the minimum required for statistical analysis of results, might also be required under regulations for registering a pesticide or similar category of chemical.

5.1 Properties, Labelling, and Storage of Sample

Information should be obtained on the properties of the chemical to be tested, including water solubility, vapour pressure, chemical stability, dissociation constants, and biodegradability. Data sheets on safety aspects of the substance should be consulted, if available. Such information on the chemical will assist in determining any special requirements for handling and testing it, for example the possible need for a ventilated facility. Information on solubility and stability in seawater and fresh water will also be useful for interpreting test results. If aqueous solubility is in doubt or problematic, acceptable procedures used previously for preparing aqueous solutions of the chemical should be obtained and reported. Other available information such as structural formula, degree of purity, nature and percentage of significant impurities, presence and amounts of additives, and n-octanol:water partition coefficient should be obtained and recorded. An acceptable analytical method should also be known for the chemical in water at concentrations intended for the test, together with data on precision and accuracy.

Chemical containers must be sealed and coded or labelled upon receipt to indicate at least the chemical name, supplier, and date received. Storage conditions are to be dictated by the nature of the chemical, and often include temperature restrictions and the need for protection from light. Standard operating procedures for chemical handling and storage should be followed.

5.2 Preparing Test Solutions

Test solutions of the chemical should be prepared, if possible, by adding aliquots of a stock solution made up in control/dilution water. If fresh or distilled water was used to make the stock solution, brine or ocean salts would normally be added to the test concentrations to bring them to the standard salinity (Subsections 2.3.4 and Section 3.4). The concentration and stability of the test chemical in the stock solution should be determined before the test. Stock solutions subject to photolysis should be shielded from light, and unstable solutions must be newly prepared as necessary.

For chemicals that do not dissolve readily in water, stock solutions may be prepared using the generator column technique (Billington *et al.*, 1988; Shiu *et al.*, 1988). Ultrasonic dispersion may be used but is less desirable since it can produce droplets that differ in size and uniformity, some of which might migrate towards the surface of the liquid, or vary in biological availability, creating variations in toxicity. Organic solvents, emulsifiers, or dispersants should not be used to increase chemical solubility except in instances where they might be formulated

with the test chemical for its normal commercial purposes. If used, an additional control solution should be prepared containing the same concentration of solubilizing agent as in the most concentrated solution of the test chemical. Such agents should be used sparingly, and should not exceed 0.1 mL/L in any test solution. If solvents are used, the preferred ones (U.S. EPA, 1985a; ASTM, 1990) are triethylene glycol and dimethyl formamide. Methanol, ethanol, and acetone could be used but are more volatile.

5.3 Control/Dilution Water

Control/dilution water may be reconstituted seawater, the laboratory's supply of natural "uncontaminated" seawater, or a sample of particular receiving water if there is special interest in a local situation. The choice of control/dilution water depends on the intent of the test.

Reconstituted seawater may be used if a high degree of standardization is required, such as for measuring toxicity of a chemical relative to values derived elsewhere for this chemical and others. There should be one or more salinities common to all tests and used for all dilutions and as the control water as described in Subsection 4.3.2 (i.e., salinity in all concentrations within 1 g/kg of the control, in the range 28 to 34 g/kg).

If the toxic effect of a chemical on a particular receiving water is to be assessed, sample(s) of the receiving water could be used as the control/dilution water by taking them from an area that was not contaminated by the chemical. Examples of such situations include appraisals of real or

potential spills of chemical(s) or intentional applications of a chemical such as spraying of a pesticide. The laboratory supply of natural seawater, or reconstituted water, may also be used as a partial simulation of the receiving water, especially if there is already an interfering toxicity in the receiving water, or its collection and use is impractical (see Subsection 4.1.1). The laboratory seawater in which adults are held is also appropriate for use in other instances such as preliminary or intra-laboratory assessment of chemical toxicity.

If information is desired on the influence of salinity on toxicity of the chemical under investigation, separate tests should be conducted concurrently at two or more salinities. However, it should be kept in mind that salinities outside the 28 to 34 g/kg range might in themselves affect success of fertilization.⁴⁵ Control/dilution water for such tests should be from a single source, either reconstituted seawater (Subsection 2.3.4) or natural seawater diluted appropriately with "uncontaminated" fresh water.

5.4 Test Observations and Measurements

In addition to the observations on toxicity described in Section 4.4, there are other observations and measurements to be made during testing with chemicals.

During preparation, each solution should be examined for evidence of chemical presence and change, such as odour, colour, opacity, precipitation, or flocculation. Any observations should be recorded.

⁴⁵ Outside the limited range favourable for gametes, the effect of salinity on toxicity of a substance would be evaluated more successfully by using some other test with a euryhaline organism, rather than the fertilization assay.

It is desirable and recommended that aliquots of test solutions be analyzed to determine the concentrations of chemicals to which gametes are exposed, in at least the high, medium, and low concentrations, and the control(s).⁴⁶

The aliquots should be preserved, stored, and analyzed according to best proven methodologies for measuring the chemical in aqueous solution. Toxicity results for any tests in which concentrations are measured should be calculated and expressed in terms of those measured concentrations, unless

there is good reason to believe that the chemical measurements are not accurate.

5.5 Test Endpoints and Calculations

The statistical endpoints for tests performed using chemicals will be the standard ones described in Subsections 4.5.2 and 4.5.3, i.e., NOEC/LOEC, or ICp, or both.

If a solvent control is used, the test is rendered invalid if the fertilization success is decreased significantly from that for the control using only water.

⁴⁶ Such analyses need not be undertaken in all instances, due to analytical limitations, cost, or previous data indicating chemical stability under conditions similar to those in the test. Chemical analyses are particularly advisable if the test solutions are aerated, the test substance is volatile, insoluble, or precipitates out of solution, or if the test chemical is known to sorb to the material(s) of the test vessels (U.S. EPA, 1985a). Some situations (e.g., testing of pesticides for purposes of registration) can require the measurement of chemical concentrations in test solutions.

Section 6

Specific Procedures for Testing Samples of Effluent, Leachate, and Elutriate

This section gives particular instructions for testing samples of effluent, leachate, and elutriate, in addition to the procedures listed in Section 4.

6.1 Sample Collection, Labelling, Transport, and Storage

Containers for transportation and storage of samples of effluent, leachate, or elutriate must be made of nontoxic material. Glass or Teflon™-coated containers are preferred as they are inert and reduce sorption of chemicals. Polyethylene or polypropylene containers manufactured for transporting drinking water are less desirable but may also be used. The containers must either be new or thoroughly cleaned and rinsed with uncontaminated water. They should also be rinsed with the sample to be collected. Containers should be filled to minimize air space.

Most tests with effluent, leachate, or elutriate will be performed "off-site" in a laboratory. Testing of effluent and leachate samples should commence within 24 h of sampling whenever possible, and must commence no later than 72 h after sampling. Samples of sediment collected for aqueous extraction and subsequent testing of the elutriate should also be tested as soon as possible; extraction procedures should begin within two weeks of sampling (preferably within one week), and testing must start no later than six weeks after collection (Environment Canada, 1993). Procedures given in Environment Canada (1993) for the preparation of elutriates should be followed. Testing of

such elutriates must commence within 72 h of their preparation, or as specified in a regulation or protocol.

A two-litre sample is adequate for an off-site multi-concentration test (e.g., concentrations of 100, 50, 25, 12.5, 6.3, 3.2, 1.6%), associated routine chemical analysis, and any necessary adjustments or repeat tests. Smaller amounts are required for single-concentration tests (Subsection 4.5.4). Upon collection, each sample container must be filled, sealed, and labelled or coded. Labelling should include at least sample type, source, date and time of collection, and name of sampler(s). Unlabelled or uncoded containers arriving at the laboratory should not be tested. Nor should samples arriving in partially-filled containers be routinely tested, because volatile toxicants escape into the air space. However, if it is known that volatility is not a factor, such samples might be tested at the discretion of the investigator.

All samples of effluent or leachate should be kept cool (1 to 7°C, preferably $4 \pm 2^\circ\text{C}$) throughout their period of transport and storage. Upon collection, warm ($>7^\circ\text{C}$) samples should be cooled to 1 to 7°C with ice or frozen gel packs. As necessary, gel packs or other means of refrigeration should be used to assure that sample temperature remains within 1 to 7°C during transit. Samples must not freeze during transport.

Upon arrival at the laboratory, an aliquot of effluent or leachate required at that time may be adjusted immediately or overnight to the test temperature and used in the test. Any

remaining portion(s) of sample held for possible additional testing should be stored in darkness in sealed containers at $4 \pm 2^\circ\text{C}$.

Temperature conditions should also be as previously indicated for transportation and storage of elutriates, as well as for samples intended for extraction and subsequent testing of elutriate, unless otherwise specified.

6.2 Preparing Test Solutions

Samples in the collection containers must be agitated thoroughly just before pouring to ensure the re-suspension of settleable solids. Sub-samples (i.e., a sample divided between two or more containers) must be mixed together to ensure their homogeneity. If further sample storage is required, the composited sample (or a portion thereof) should be returned to the sub-sample containers and stored (Section 6.1) until used.

Samples may be passed through a $5\text{-}\mu\text{m}$ filter before use, and although optional, that treatment should be regarded as the standard practice. The presence of high concentrations of suspended inorganic or organic solids in a sample might inhibit fertilization or damage the gametes directly. High concentrations of biological solids in certain types of treated wastewaters can also contribute to sample toxicity due to ammonia and/or nitrite production (Servizi and Gordon, 1986). Filtration would also remove filter-feeding organisms that might be present in treated wastewater, or other organisms that might eat gametes.

Filtration will remove some suspended solids that are characteristic of the sample and might otherwise contribute part of the toxicity or modify the toxicity. If there is concern about the removal of toxicity by the

filtration process, two tests should be carried out concurrently, one without filtration and a second with filtered sample. The two tested aliquots should be treated in an identical manner, except for the filtration.

6.3 Control/Dilution Water

Tests with samples of effluent or leachate, intended to assess compliance with regulations, should use as the control/dilution water, either the laboratory seawater normally supplied to the adults, or a sample of the receiving water. Because results could be different for the two sources of water, the objectives of the test must be decided before a choice is made. Shipping difficulties and costs should also be considered; the use of receiving water for dilutions and controls increases the volume of liquid to be shipped, although that might not be a major factor for this small-scale assay.

The use of "upstream" receiving water as the control/dilution water can be desirable if information is required on the potential toxic effect of an effluent, leachate, or elutriate on a particular receiving water (see rationale in Subsection 4.3.2). An important example of such a situation would be testing for sublethal effect at the edge of a mixing zone, under site-specific regulatory requirements. Conditions for the collection, transport, and storage of such receiving-water samples should be as described in Section 6.1. Any sample of receiving water used as the control/dilution water for testing effluents or leachates should be filtered according to the standard recommendations for all control/dilution water, at $1.0\text{ }\mu\text{m}$ (Subsection 4.1.1). If a sample of receiving water is to be used as control/dilution water, a separate control solution should be

prepared using the laboratory seawater supplied to the adults.

Tests requiring a high degree of standardization should use reconstituted seawater as control/dilution water. An example of such a situation might be a test intended to compare the toxicity of a particular effluent, leachate, or elutriate with that of samples collected and/or tested elsewhere.

As a standard procedure for assessing toxic substances in the wastewater, the salinity of all test and control solutions should be adjusted to the same value, usually by adjusting an aliquot of the sample as described in Subsection 4.3.2 (making test solutions within 1 g/kg of each other in the range 28 to 34 g/kg). Procedures using brine or dry commercial sea salt are recommended, as described in Subsection 2.3.4. If it is desired to assess the total effect of the wastewater including its low (or high) salinity, for regulatory or compliance purposes, the test could be run without adjustment of salinity. An evaluation could be made for both purposes (presence of toxic substances, and compliance) by running the adjusted test and a second test with unadjusted salinities, and/or a set of salinity controls (Subsection 4.1.4).

6.4 Test Observations and Measurements

Success of fertilization should be observed as in Section 4.4.

Colour, turbidity, odour, and homogeneity, i.e., the presence of floatable or settleable material, should be observed in the sample of effluent, leachate, or elutriate at the time of preparing test solutions. A record should

be made of overt changes upon dilution with water or during the test, such as precipitation, flocculation, foaming, odour, and change in colour or turbidity.

For effluent samples having appreciable solids content, it is desirable to measure total suspended and settleable solids (APHA *et al.*, 1989) upon receipt, as part of the overall description of the effluent, and as sample characteristics that might influence the results of the toxicity test.

6.5 Test Endpoints and Calculations

Tests for monitoring and for compliance with regulatory requirements should normally include, as a minimum, three or more undiluted portions of the sample, and three or more replicate control solutions. Test procedures for regulatory compliance could specify that a single concentration (100% sample unless otherwise specified) be used, or might require determination of the NOEC/LOEC and/or ICp.

Toxicity tests can have other objectives such as determination of in-plant sources of toxicity, or toxicity changes resulting from waste treatment or process changes. Such tests might be multi-concentration tests or single-concentration tests (100% or an appropriate dilution, plus a control). Single-concentration tests are often cost-effective for determining the presence or absence of measurable toxicity or as a method for screening a large number of samples for relative toxicity. Endpoints for these tests would again depend on the objectives of the undertaking, but could include arbitrary "pass" or "fail" ratings, or percent reduction in fertilization at a specified concentration. Items in Section 4.5 provide instructions that are relevant here, on statistical analysis and reporting of results from a set of tests on different samples, each tested at only one concentration.

Section 7

Specific Procedures for Testing Receiving-water Samples

Instructions for testing samples of receiving waters, additional to those provided in Section 4, are given here.

7.1 Sample Collection, Labelling, Transport, and Storage

Procedures for the labelling, transportation, and storage of samples should be as described in Section 6.1. Testing of samples should commence as soon as possible, preferably within 24 h, and no later than 72 h after sampling.

7.2 Preparing Test Solutions

Samples in the collection containers should be agitated before pouring to ensure their homogeneity. Compositing of sub-samples should be as described in Section 6.2.

Samples may be filtered at 5 μm before use. If there is concern about changes in toxicity from the filtering process or removal of solids, both filtered and unfiltered tests could be run as described in Section 6.2.

7.3 Control/Dilution Water

For receiving-water samples collected in the vicinity of a wastewater discharge, chemical spill, or other point-source of possible contamination, "upstream" water may be sampled concurrently and used as control water and diluent for the "downstream" samples. Discussion in Subsection 4.1.1 is relevant here, on the implications and possible effects of using such water for the control and for dilution. This

control/dilution water should be collected as close as possible to the contaminant source(s) of concern, but outside its zone of influence. Water current or dispersal tracer studies might be necessary to establish an acceptable sampling location. All control/dilution water should be filtered (Subsection 4.1.1, and Sections 6.2 and 6.3).

If "upstream" water is used as control/dilution water, a separate control solution should be prepared using the laboratory seawater that is normally supplied to the adult echinoids. Test conditions and procedures for preparing and evaluating each control solution should be identical, and as described in Sections 4.1 and 5.3.

Logistic constraints, lack of on-site information, expected toxic effects, or other site-specific practicalities might prevent or rule against the use of "upstream" water as the control/dilution water. In such cases, the laboratory seawater supply normally used for holding adults should be used as control/dilution water. This water may be adjusted in salinity to partially simulate "upstream" water (Subsection 4.1.1), but the salinity limitations of this echinoid assay preclude major manipulations.

7.4 Test Observations and Measurements

Observations of sample and solution colour, turbidity, foaming, precipitation, etc. should be made as described in Section 6.4, both during preparation of solutions and subsequently during the tests. These are in

addition to the primary toxicity observations described in Section 4.4.

7.5 Test Endpoints and Calculations

Statistical endpoints for tests using samples of receiving water should be consistent with the options and approaches identified in Sections 4.5 and 6.5, and would again be based on success in fertilization compared to the control(s).

Tests for monitoring and compliance should normally include, as a minimum, three or more undiluted portions of the sample, and

three or more replicate control solutions. If toxicity of receiving-water samples is likely, and information is desired concerning the degree of dilution necessary to permit normal fertilization in echinoids, a full test to determine NOEC/LOEC and/or IC_p should be conducted as outlined in Section 4, with undiluted sample as the highest concentration in the series tested.

Certain sets of tests might use a series of samples such as seawater from a number of locations, each tested at full strength only. Statistical testing and reporting of results for such tests should follow the procedures outlined in Subsections 4.5.2 and 4.5.3.

Section 8

Specific Procedures for Testing Samples of Sediment and Similar Substances

General instructions are given here for testing liquids derived from samples of sediment or similar solids such as sludges and soils. These are in addition to the general instructions provided in Section 4. In this section, the word "sediment" is used for convenience but should be taken to include other similar solid substances such as soils and industrial or municipal sludges, which might contribute pollutants to natural waters or require testing for other reasons.

8.1 General Aspects of Procedure

Assessing the toxicity of sediments is becoming widely recognized as an important part of environmental protection, notably when considering ocean dumping, such as under Part VI of the *Canadian Environmental Protection Act*.

The echinoid fertilization assay is suitable for testing the toxicity of liquids derived from sediments. It provides a rapid method for comparing extracts of contaminated sediments (Long *et al.*, 1990). Tests on sediment-derived liquids showed that an echinoderm embryo test and the bacterial Microtox test were the most sensitive of seven sediment tests evaluated (Pastorok and Becker, 1989). Sublethal toxicity tests including a fertilization assay using *Arbacia* and interstitial water from sediments, were considerably more sensitive than a standard test on the whole sediment using amphipods (Carr and Chapman, 1992).

General guidance is given here on application of the echinoid fertilization assay

for testing liquids derived from sediments. It is not the purpose of this report to provide instructions for carrying out a field survey of sediments, sampling them, or extracting aqueous or other substances from them. Detailed guidance for the collection, handling, transport, and storage of sediment samples (Subsection 8.1.1) is provided in Environment Canada (1993). Also in Environment Canada (1993) are procedural details for the extraction of liquids (i.e., pore water, elutriate, or solvent extract; (Subsection 8.2) from sediments for subsequent toxicity tests and chemical analyses. This recent guidance document should be consulted and followed, in addition to the guidance provided here. Detailed information regarding the basis of such guidance can be found in books (e.g., Mudroch and Macknight, 1991) and in primary literature cited in Environment Canada (1993) or related reviews of this subject matter (e.g., Geisy and Hoke, 1989; McLeay and Sprague, 1991).

This fertilization assay is not suitable for testing the sediments themselves (i.e., the solids), but is useful for liquids derived from those solids, whether leachate, elutriate, solvent extract, or interstitial water. For economy of wording, the following sections sometimes refer to "testing sediments", but that should be taken as testing the liquids derived from the sediments.

8.1.1 Sample Labelling, Transport, and Storage

General procedures for labelling, transporting, and storing sediment samples

should be as described in Section 6.1. Temperature limits are those described in Section 6.1, and samples must not freeze or partially freeze, or be allowed to dry (ASTM, 1991b; Environment Canada, 1993).

For the liquids derived from sediments, containers and handling procedures should be the same as those given in Section 6.1 for elutriates. If a non-aqueous solvent has been used to extract substances, a glass container should be used to store the liquid, so that it will not be affected by the solvent or leach substances into the sample.

Testing of samples should start as soon as possible after collection. Extraction procedures should begin within two weeks of sampling, and preferably within one week. Testing must start no later than six weeks after collection of samples (Environment Canada, 1993).⁴⁷ Testing of the liquid obtained from sediments should begin within 72 h of making such preparations (Environment Canada, 1993), or as specified in a regulation or other designated procedure.

8.1.2 Preparing Sample

Depending on the nature of the sample and the objectives of the test, homogenization of samples might or might not be required before testing. If mixing is carried out, it must be thorough. Sub-samples (i.e., a sample divided between two or more containers) must be mixed together. If further sample storage is required, the composited sample, or a portion of it, should be returned to the sub-sample containers and stored.

8.1.3 Observations and Measurements on Sample

Observations of the colour, turbidity, foaming, precipitation, etc. should be made on both the sediment and any liquid derived from it, during preparation of test solutions, as described in Section 6.4.

8.1.4 Control or Reference Sediments

One or more samples of control or reference (unpolluted) sediment must be assessed in the same manner as the sediment under investigation. Although the test procedures include a blank or control which does not contain substances from the sediment being studied, experience indicates that such a control might not be sufficient for an acceptable evaluation of toxicity. Using other kinds of tests, Environment Canada and other laboratories have frequently recorded apparent toxic effects with unpolluted sediments. Accordingly, one or more control or reference ("clean") sediments should be included as a sample, with each test of a sediment or series of sediments, to help establish a baseline or "normal" level. It would be desirable to establish a standard, clean "reference sediment" for this purpose, or a series of reference sediments of differing characteristics that could be matched with those of the sediments being tested. The control or reference sediment(s) should be similar in general physical and chemical characteristics to the sediment(s) being investigated. In particular, an attempt should be made to match the distribution of particle sizes and organic/inorganic balance (ASTM, 1991a; 1991b; McLeay and Sprague, 1991).

⁴⁷ The toxicity and geochemistry of contaminated sediments from Hamilton Harbour were reported to change with storage for longer than one week, although the data supporting that statement were not provided (Brouwer *et al.*, 1990). Testing within two weeks conforms with current standardization in U.S. procedures (ASTM, 1991b). A maximum permissible storage time of six weeks was included in draft reports of Environment Canada (1990e; 1990f) in view of practical difficulties for shorter times, including time required if initial chemical analyses are to be performed.

There is no single procedure for making use of the results from the control or reference sediment. If the control sediment shows no toxicity, then results for the test sediment (i.e., the one under investigation) are accepted as valid. If the reference sediment shows toxicity, no standard method appears to have been developed, as yet, to adjust the results for the test sediment.⁴⁸

Caution should be exercised in interpreting findings for the test substance. It would be desirable to test for significant differences between results for the reference and test sediments. Such tests could be carried out with guidance from a statistician, if paired observations or replicate tests were available.

8.2 Testing Liquids Extracted from Sediments and Similar Solids

Toxicants from sediments or soils can enter an aqueous phase and affect organisms in natural waters. The aqueous phase might be a liquid derived from a soil or sediment (e.g., interstitial water), or a liquid used to treat the sample and extract potential toxicants (e.g., an elutriate).

A liquid obtained from sediment for toxicity testing would be expected to fall into one of four broad categories.

- (1) Interstitial water, i.e., that which fills the spaces between particles, and could exchange with the overlying water making up the estuary, bay, etc. It is normally obtained from a sediment by centrifuging or squeezing it (ASTM, 1991b).

- (2) Water that is essentially fresh water, used to obtain an aqueous extract of substances from the sediment (i.e., elutriate), for example by shaking a sample with added clean water. This category is not very appropriate for the echinoid fertilization assay.
- (3) Control/dilution water or other clean water which has a salinity equivalent to seawater, used to obtain an aqueous extract as in (2).
- (4) Solvents other than water (e.g., organic solvents), used to remove substances from the sample of sediment (Schiewe *et al.*, 1985; True and Heyward, 1990).

The water of the first three categories could be tested as a normal liquid sample, following the universal procedures given in Section 4 and the procedures for effluents, leachates, and elutriates as given in Section 6.

For the fourth category, solvents, the preferred option is to have the same concentration of solvent in each of the test vessels. The control/dilution water to be used in the test is brought to the same concentration of solvent as that in the highest concentration of sample that will be tested. Lower concentrations are prepared using the modified control/dilution water, so that the solvent effect, if any, should be the same in all vessels. There should be a control without solvent, as well as the one with solvent. It would be desirable to run a separate test to determine the NOEC/LOEC and/or IC_p of the solvent.

⁴⁸ Brouwer *et al.* (1990) incorporated such a control by expressing the performance measured for the test sediment as a percentage of that from the control (reference) sediment, using a bacterial test procedure. However, only one concentration of each was tested, and the technique does not appear to have been extended to a multi-concentration test.

8.2.1 Preparing Test Substances

Specific guidance is given in Environment Canada (1993) for the extraction of liquids (i.e., pore water, elutriate, or solvent extract) from sediment. This guidance should be consulted and followed when preparing sediment extracts for echinoid fertilization assays.

Compositing of "sub-samples" of liquid obtained from the sediment (e.g., successive extractions) should be as described in Section 6.2. Sub-samples would not be composited if the relative toxicity of successive extractions was to be ascertained. Filtration at 5 μm is recommended, as for standard samples of natural seawater. The pH and dissolved oxygen content of the sample should be checked with regard to the limits in Subsections 4.1.2 and 4.1.3.

Once the liquid has been obtained, test concentrations are prepared in the standard manner (Subsection 4.1.2). As in testing effluents, leachates, and elutriates, there could be a single-concentration test (plus control) for regulatory purposes, or a multi-concentration test to determine the NOEC/LOEC or IC_p, or both (Section 6.2). The procedures for obtaining and testing liquid from samples of control or reference

sediment should be identical to those used for the test sediment.

8.2.2 Control/Dilution Water

If the sediment sample is marine or estuarial, and the water derived from it is essentially seawater in the standard range of salinity for this test (28 to 34 g/kg), the Universal procedures of Section 4 would be followed.

For freshwater samples derived from sediment, or if the water derived from the sediment has salinity lower than that of full-strength seawater, i.e., similar to estuarial water, its salinity would normally be adjusted upwards. The standard procedure would be to adjust the salinity of all test and control solutions to the same value (within 1 g/kg of each other in the range 28 to 34 g/kg), usually accomplished by adjusting an aliquot of the sample as described in Subsection 4.3.2. Procedures using brine or dry commercial sea salt are recommended, as outlined in Subsection 2.3.4.

8.2.3 Endpoints and Calculations

Endpoints for tests with liquids derived from sediment should be consistent with the options and approaches identified in Sections 4.5 and 6.5.

Section 9

Reporting Requirements

The test report should describe the materials and methods used, as well as the results. A reader should be able to establish from the report whether the conditions and procedures rendered the results acceptable for the use intended.

Procedures and conditions that are common to a series of ongoing tests (e.g., routine toxicity tests for monitoring or compliance purposes), and consistent with specifications in this report may be referred to by citation or by attachment of a general report that outlines standard laboratory practice. Where choices exist, the approach selected should be specified. The general report should convey the procedural information included in Sections 9.2 to 9.5. A report giving the findings of an individual test should contain the information indicated in Sections 9.1, 9.6 and 9.7. Specific monitoring programs might require other selected items in the report (e.g., procedures and results for tests requiring pH adjustment or modified aeration/oxygenation). Other details pertinent to the conduct and findings of the test, that are not conveyed by the reports, should be kept on file by the laboratory, so that the appropriate information can be provided if an audit is required.

9.1 Test Substance

- sample type, source, and description (chemical, effluent, leachate, elutriate, or receiving water; sampling location and method; specifics regarding nature, appearance and properties, volume and/or weight);

- information on labelling or coding of the test substance;
- details on manner of sample collection, transport and storage (e.g., batch, grab or composite sample, description of container, temperature of sample upon receipt and during storage);
- identification of person(s) collecting and/or providing the sample; and
- dates and times for sample collection, receipt at test facility, start and end of definitive test.

9.2 Test Organisms

- species and source;
- description of holding conditions for adults (facilities, lighting, water source and quality, water pre-treatment, water exchange rate and method, density of animals in tanks, temperatures in those tanks, food type and frequency of feeding, disease incidence); and
- weekly percentage of mortalities among the adults being held.

9.3 Test Facilities and Apparatus

- name and address of test laboratory;
- name of person(s) performing the test;
- description of systems for regulating temperature within test facility; and

- description of test vessels (usual name, size, shape, type of material).

9.4 Control/Dilution Water

- type(s) and source(s) of water used as control and dilution water;
- type and quantity of any chemical(s) added to control or dilution water;
- sampling and storage details if the control/dilution water was "upstream" receiving water;
- water pre-treatment (temperature adjustment, de-gassing, aeration rates, duration, etc.); and
- measured water quality variables (Subsection 2.3.4) before and/or at time of commencement of toxicity test.

9.5 Test Method

- brief mention of method used if standard (e.g., as per this document), and options selected;
- design and description if specialized procedure or modification of standard method;
- procedure used in preparing stock and/or test solutions of chemicals;
- any chemical analyses of test solutions and reference to analytical procedure(s) used; and
- use of preliminary or range-finding test.

9.6 Test Conditions

- statement on aeration of sample before the test (if any, give rate, duration, and manner);
- number, concentration, volume, and depth of test solutions including controls, and number of replicates;
- estimated number of sperm per vessel and sperm:egg ratio;
- general nature of lighting of test facility;
- description of any test solutions receiving pH adjustment or filtration, including procedure;
- any chemical measurements on test solutions (e.g., chemical concentration, suspended solids content);
- temperature, salinity, pH, and dissolved oxygen (mg/L and % saturation) as measured in aliquots of control, and high, medium and low test solutions at start; and
- conditions and procedures for measuring the IC_p or NOEC/LOEC of the reference toxicant(s).

9.7 Test Results

- appearance of test solutions and changes noted during test;
- numbers of fertilized and unfertilized eggs counted for each vessel at the end of the test, and percent fertilized;
- results for range-finding test, if conducted;
- the NOEC/LOEC or IC_p, or both, as measures of fertilization success;

Minimum Significant Difference in % fertilization, if NOEC/LOEC are determined; the statistical test(s) used, and any transformation of data that was required; and

- the results of the relevant toxicity test(s) with the reference toxicant(s), together with the geometric mean value (± 2 SD) for the same reference toxicant(s) as derived at the test facility in previous tests.

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Appendix A

Members of the Inter-Governmental Aquatic Toxicity Group*

Federal, Environment Canada

P.G. Wells
EP, Dartmouth, N.S.

P. Jackman
St. John's, Nfld.

K.G. Doe
Dartmouth, N.S.

W.R. Parker
Dartmouth, N.S.

S.J. Wade
Dartmouth, N.S.

J.D. Vaughan
Dartmouth, N.S.

N. Bermingham
Longueuil, Quebec

C. Blaise
Longueuil, Quebec

D. St. Laurent
Longueuil, Quebec

G. Elliott
Edmonton, Alberta

R.G. Watts
North Vancouver, B.C.

S.G. Yee
North Vancouver, B.C.

D. Moul
North Vancouver, B.C.

K.E. Day
National Water Research Inst.
Burlington, Ont.

A.K. Kwan
National Water Research Inst.
Burlington, Ont.

C. Boutin
Canadian Wildlife Service
Hull, Quebec

J. Osborne
Office of Waste Management
Hull, Quebec

M. Bonnell
EcoHealth Branch
Hull, Quebec

D. MacGregor
Regulatory Affairs & Program Integration
Branch
Hull, Quebec

R.P. Scroggins
Technology Development Branch
Hull, Quebec

G.A. Sergy
Technology Development Branch
Edmonton, Alberta

Federal, Fisheries & Oceans

R. Stevens
Oceanography & Contaminants Branch
Ottawa, Ont.

Provincial

C. Bastien
Ministère de l'Environnement du Québec
Sainte Foy, Quebec

S.G. Abernethy
Ont. Ministry of Environment
Rexdale, Ont.

C.M. Neville
Ont. Ministry of Environment
Rexdale, Ont.

D.G. Poirier
Ont. Ministry of Environment
Rexdale, Ont.

I.R. Smith
Ont. Ministry of Environment
Rexdale, Ont.

G.F. Westlake (Chairperson)
Ont. Ministry of Environment
Rexdale, Ont.

B. Bayer
Manitoba Min. Environment,
Winnipeg, Manitoba

J. Somers
Alberta Environment Centre
Vegreville, Alberta

K. Smiley
Alberta Environment Centre
Vegreville, Alberta

S.H. Horvath
B.C. Ministry of Environment
Vancouver, B.C.

G. van Aggelen
B.C. Ministry of Environment
North Vancouver, B.C.

Appendix B

Conservation & Protection, Regional and Headquarters Offices

Headquarters

351 St. Joseph Boulevard
Place Vincent Massey
Hull, Quebec
K1A 0H3

Atlantic Region *

15th Floor, Queen Square
45 Alderney Drive
Dartmouth, Nova Scotia
B2Y 2N6

Quebec Region

1141 Route De L'Eglise
P.O. Box 10100
Sainte Foy, Quebec
G1V 4H5

Ontario Region

25 St. Clair Ave. East, 6th Floor
Toronto, Ontario
M4T 1M2

Western and Northern Region

Room 210, Twin Atria # 2
4999 - 98 Avenue
Edmonton, Alberta
T6B 2X3

Pacific and Yukon Region

224 Esplanade Street
North Vancouver, British Columbia
V7M 3J7

* The statistical programs of TOXSTAT and BOOTSTRP are available from the Laboratory Division at this address by providing a formatted computer diskette.

Appendix C

Review of Procedural Variations Used by Previous Authors and Groups for Fertilization Assays Using Sea Urchins and Sand Dollars

Based on documents available to the authors in March, 1992. The following elements of procedure are omitted because they were common to all tests, or could be easily adapted to all methods covered here.

- **Static tests** - All exposure and fertilization was in small vessels without renewal of solutions.
- **Test substance** - All methods could be used for pure chemicals, formulations, wastewaters, or samples of seawater, by adjusting salinity as is common practice in the methods reviewed.
- **Endpoints** - The usual endpoint was reduced fertilization compared to control. All methods appear suitable for estimating IC₅₀ and NOEC/LOEC by usual statistical techniques.

Explanation of authors or originating agency.

Beak 1988, is the Canadian consulting company, listed in the references.

EVS 1989 is the Canadian consulting company, listed in the references.

B.C. MOE 1990 is British Columbia Ministry of Environment, and includes van Aggelen (1988).

IGATG 1991 includes that reference and Jonczyk *et al.*, (1991).

Dinnel *et al.* 1987, with co-authors, represents a major school or approach in echinoid testing.

U.S. EPA 1988 is in the book of methods published by the Cincinnati office of EPA.

ASTM 1990 is a subcommittee developing a standard method, chairman G.A. Chapman.

NCASI 1991 and 1992 are in reference list; a scientific group sponsored by pulp and paper industry.

U.S. EPA (Pac. 91) is in reference list as Chapman (1991), a Pacific-coast method for interlaboratory comparisons, which prompted documents from U.S. consultants (see following).

U.S. EPA (Pac. 92) is Chapman (1992) in reference list, a draft Pacific-coast method of EPA.

Kobayashi 1971 represents the early methods used by this productive researcher.

Kobayashi 1984 represents a later synopsis of methods by this researcher.

S. Calif. Project was a regional pollution research agency, in references as Oshida *et al.*, 1981.

Nacci *et al.* 1986 is a publication cited by others as a source of methods.

Cherr *et al.* 1987 are authors from the Bodega Marine Lab.

BML 1991 is in references, and is Bodega Marine Lab, part of Univ. of California.

ERCEES 1990 is a U.S. consulting company in California and is in reference list.

MECAS 1990 is a U.S. consulting company in California and is in reference list.

NWAS 1990 is a U.S. consulting company on the west coast and is in reference list.

The order of listing is (1) Canadian laboratories, (2) major committees, government agencies, laboratories and schools (which happen to be in the United States), and (3) consulting laboratories and major authors. Detailed methods of Pagano and colleagues were not clear from papers and have been omitted.

Abbreviations:

lab.	=	laboratory
N.I.	=	not indicated
c/d water	=	control/dilution water
Pac.	=	Pacific
reconst.	=	reconstituted
s.u.	=	sea urchin(s)
s.d.	=	sand dollar(s)

1. Species and Availability of Adults

Document	Species, Information Given on Location, Collection, Spawning Season
Beak 1988	<i>Lytechinus pictus</i> Californian urchin, purchased. Spawning condition year-round.
EVS 1989	<i>Strongylocentrotus droebachiensis</i> green sea urchin, Canadian Atlantic, Pacific, Arctic. Said to spawn March to April. <i>S. purpuratus</i> Pacific purple sea urchin. Collect from clean locations or purchase. Spawns Dec. to March. <i>S. droebachiensis</i> as above. <i>S. franciscanus</i> red sea urchin, Pacific. Spawns April-May. <i>Dendraster excentricus</i> "eccentric sand dollar" of the Pacific, said to spawn late spring and summer
B.C. MOE 1990	<i>D. excentricus</i> eccentric sand dollar as above, but said to spawn June to Nov.
IGATG 1991	<i>S. droebachiensis</i> as above, but spawns Feb. to March or April. <i>L. pictus</i> , as above.
Dinnel <i>et al.</i> 1987	<i>S. purpuratus</i> Ripe Dec. to March, longer in lab. <i>S. droebachiensis</i> as above. Ripe Jan. to April, longer in lab. <i>S. franciscanus</i> as above. <i>D. excentricus</i> as above. Ripe May to October.
U.S. EPA 1988	<i>Arbacia punctulata</i> "Arbacia", or Atlantic purple sea urchin. May be purchased.
ASTM 1990	<i>A. punctulata</i> as above. <i>D. excentricus</i> as above. <i>S. purpuratus</i> as above. <i>S. droebachiensis</i> as above. Other species may be used if necessary.
NCASI 1991, 1992	<i>D. excentricus</i> as above. Spawn all year except late Dec. to late Jan. by arranging laboratory holding conditions. <i>S. purpuratus</i> Spawns Jan. to June by lab. holding. <i>S. droebachiensis</i> as above. Also Jan. to June by lab. holding.
U.S. EPA (Pac. 91)	<i>S. droebachiensis</i> as above.
U.S. EPA (Pac. 92)	<i>S. purpuratus</i> as above.
Kobayashi 1971	<i>Hemicentrotus pulcherrimus</i> a sea urchin, Japan. Spawns Jan. to March. <i>Anthocardaris crassispina</i> a sea urchin, Japan. Spawns May to Aug. <i>Temnopleurus toreumaticus</i> a sea urchin, Japan. Spawns July to Oct. <i>Pseudocentrotus depressus</i> a sea urchin, Japan. Spawns Oct. to Nov.
Kobayashi 1984	Same as Kobayashi 1971 except <i>T. toreumaticus</i> not mentioned.
S. Calif. Project	<i>S. purpuratus</i> as above. Collect by hand.
Nacci <i>et al.</i> 1986	<i>A. punctulata</i> as above.
Cherr <i>et al.</i> 1987	<i>S. purpuratus</i> as above.
BML 1991	<i>S. purpuratus</i> as above.
ERCEES 1990	<i>S. purpuratus</i> as above. Collect or purchase. <i>A. punctulata</i> as above. Collect or purchase. <i>Lytechinus</i> sp. as above. Collect or purchase. <i>D. excentricus</i> as above. Collect or purchase.
MECAS 1990	N.I.
NWAS 1990	<i>S. purpuratus</i> as above. <i>D. excentricus</i> as above. Purchased as necessary.

2 Holding Adults in the Laboratory

Document	Duration	Water	Feeding
Beak 1988	5 d	reconstituted seawater	romaine lettuce? s.u. given macroalga
EVS 1989	≤ 9 wk	flowing seawater at 0.1 L/min per shallow tray, or static with monthly replacement	s.u. with brown macroalga s.d. with eel grass
B.C. MOE 1990	N.I.	unfiltered flowing seawater	s.d. not fed
IGATG 1991	≥ 7 d	green s.u., flowing seawater white s.u. in reconst. seawater	green s.u., brown macroalga, white s.u., romaine lettuce
Dinnel <i>et al.</i> 1987	N.I.	flowing seawater, filtered recirculation with filter	s.u. macroalga s.d. plankton and detritus
U.S. EPA 1988	N.I.	filtered seawater, 5 L/min, for 20-L tank with 20 adults, or recirculated reconst. seawater	s.u. kelp or romaine lettuce
ASTM 1990	N.I.	reconstituted seawater, or unfiltered seawater	s.u., macroalga, R. lettuce s.d., microalgae
NCASI 1991, 1992	N.I.	unfiltered seawater, 1 to 2 L/min to 160-L tank	s.d. algal growth, flake food s.u. macroalga, romaine lettuce
U.S. EPA (Pac. 91)	N.I.	N.I.	N.I.
U.S. EPA (Pac. 92)	N.I.	filtered seawater, 5 L/min, or recirculated reconst. seawater	kelp or romaine lettuce
Kobayashi 1971	≤ 2 d	N.I.	N.I.
Kobayashi 1984	≤ 2 d	N.I.	N.I.
S. Calif. Project	N.I.	recirculated seawater	brown alga
Nacci <i>et al.</i> 1986	N.I.	N.I.	N.I.
Cherr <i>et al.</i> 1987	N.I.	flowing seawater	macroalga
BML 1991	N.I.	N.I.	N.I.
ERCEES 1990	N.I.	seawater brought in weekly	giant kelp
MECAS 1990	0 to 2 d	flowing seawater	N.I.
NWAS 1990	days/mos.	seawater, flowing or partly recirculated	s.u. kelp or lettuce s.d. plankton and detritus

3 Holding Conditions for Adults

Document	Species	Temperature (°C)	Salinity (g/kg)	Oxygen (% sat'n)	Lighting
Beak 1988	<i>Lytechinus anamesus</i>	15	30	N.I.	NI
EVS 1989	various spp s.u. <i>D. excentricus</i>	15	28 "	airstones "	constant dark photoperiod
B.C. MOE 1990		N.I.	27 to 30	N.I.	N.I.
IGATG 1991	<i>S. droebachiensis</i> <i>L. pictus</i>	9 15	30 "	N.I. "	N.I. "
Dinnel <i>et al.</i> 1987	<i>Strongylocentrotus</i> <i>D. excentricus</i>	natural seasonal	≥ 27	N.I.	N.I.
U.S. EPA 1988	<i>A. punctulata</i>	15 ± 3	30	N.I.	N.I.
ASTM 1990	<i>Strongylocentrotus</i> <i>D. excentricus</i> <i>A. punctulata</i>	8 to 10 12 to 14 15	25 to 35 " "	50 to 100% " "	N.I. N.I. high lighting
NCASI 1991, 1992	<i>Strongylocentrotus</i> , <i>D. excentricus</i>	7 to 14	N.I.	N.I.	ambient lab.
U.S. EPA (Pac. 91)	<i>S. purpuratus</i>	N.I.	N.I.	N.I.	N.I.
U.S. EPA (Pac. 92)	<i>S. purpuratus</i>	12 (10 to 14)	> 30 (32 preferred)	N.I.	N.I.
Kobayashi 1971		N.I.	N.I.	N.I.	N.I.
Kobayashi 1984		N.I.	N.I.	N.I.	N.I.
S. Calif. Project	<i>S. purpuratus</i>	12	N.I.	N.I.	N.I.
Nacci <i>et al.</i> 1986		N.I.	N.I.	N.I.	N.I.
Cherr <i>et al.</i> 1987		N.I.	N.I.	N.I.	N.I.
BML 1991		N.I.	N.I.	N.I.	N.I.
ERCEES 1990		N.I.	N.I.	N.I.	N.I.
MECAS 1990		12	N.I.	N.I.	N.I.
NWAS 1990	<i>S. purpuratus</i> , <i>D. excentricus</i>	10 ± 2	≥ 25	N.I.	12 Light 12 Dark

4 Type of Control/Dilution Water

Document	Recommended Type of Water and Treatment
Beak 1988	deionized water with sea salts
EVS 1989	clean seawater filtered at 1 μm , UV sterilization optional
B.C. MOE 1990	seawater
IGATG 1991	deionized water with sea salts, or seawater filtered at 0.45 μm
Dinnel <i>et al.</i> 1987	seawater, filtered at 5 μm , activated carbon optional, or recirc'n with filter
U.S. EPA 1988	deionized water plus sea salts or brine; seawater may be additional control
ASTM 1990	reconstituted from sea salts or formula, filtered 0.45 μm , TOC and TSS ≤ 5 mg/L, UV sterilization if pathogens likely, must achieve 70% fertilization with sperm held in water for 1 h
NCASI 1991, 1992	seawater, filtered 1 μm and UV sterilization, aerated, held 0 h
U.S. EPA (Pac. 91)	seawater, filtered 1 μm
U.S. EPA (Pac. 92)	seawater, or reconstituted, preferably from brine
Kobayashi 1971	N.I., presumed seawater
Kobayashi 1984	N.I., presumed seawater
S. Calif. Project	N.I.
Nacci <i>et al.</i> 1986	brine prepared from seawater, diluted to salinity 30 g/kg with distilled water
Cherr <i>et al.</i> 1987	seawater, filtered 0.45 μm
BML 1991	seawater, filtered and UV sterilized
ERCEES 1990	seawater, supply renewed weekly, filtered 20 μm and 5 μm
MECAS 1990	seawater, filtered 0.45 μm
NWAS 1990	seawater, unfiltered, adjusted to 32 g/kg salinity with deionized water

5 Temperature and Salinity During Test

Document	Temperature (°C)	Salinity (g/kg) and Method of Adjustment
Beak 1988	20 ±1	30 ±2
EVS 1989	15	adjust to unspecified salinity with salts if testing seawater samples, no adjustment for freshwater samples
B.C. MOE 1990	10	N.I.
IGATG 1991	20 ±1	30 ± 2
Dinnel <i>et al.</i> 1987	s.u. 8 to 10 s.d. 12 to 16	30 ±3, adjust with sea salt or deionized water
U.S. EPA 1988	20 ±1	30 ±2, adjust effluent as necessary
ASTM 1990	12, but 20 for <i>A. punctulata</i> , and ≤2°C variation between, within vessels	>25 and <32, within 1 g/kg of control, 30 recommended; adjust with brine or salts
NCASI 1991, 1992	12	30, adjust test solutions with brine or salts
U.S. EPA (Pac. 91)	12	32 ±1
U.S. EPA (Pac. 92)	12 ±1	32 ±2, adjust sample to 32
Kobayashi 1971	N.I.	N.I., adjust low-salinity samples with brine or by boiling
Kobayashi 1984	N.I.	N.I.
S.Calif. Project	N.I.	N.I., apparently not adjusted. Some tests 31 to 32.6
Nacci <i>et al.</i> 1986	N.I.	N.I.
Cherr <i>et al.</i> 1987	N.I.	N.I.
BML 1991	15	32, adjust both sample and water if necessary
ERCEES 1990	"appropriate"	N.I., adjusted if necessary with brine or deionized water
MECAS 1990	12 ±1	30 ±2, adjust test solutions with brine or spring water
NWAS 1990	12 ±1	32 ±2, adjust sample as needed with brine

6 Dissolved Oxygen and Lighting During Test

Document	Initial DO (% saturation) and Adjustment	Lighting
Beak 1988	N.I.	normal lab., nominal 1100 lux
EVS 1989	aerate samples enough to attain acceptable DO	N.I.
B.C. MOE 1990	N.I.	N.I.
IGATG 1991	N.I.	normal lab., nominal 1100 lux
Dinnel <i>et al.</i> 1987	N.I.	N.I.
U.S. EPA 1988	N.I.	normal lab., 540 to 1080 lux
ASTM 1990	90 to 100% in c/d water	N.I.
NCASI 1991, 1992	N.I.	normal lab. fluorescent
U.S. EPA (Pac. 91)	N.I.	N.I.
U.S. EPA (Pac. 92)	N.I.	normal lab., 540 to 1100 lux
Kobayashi 1971, 1984 N.I.		N.I.
S. Calif. Project	Not controlled	N.I.
Nacci <i>et al.</i> 1986	N.I.	N.I.
Cherr <i>et al.</i> 1987	N.I.	N.I.
BML 1991	N.I.	N.I.
ERCEES 1990	N.I.	N.I.
MECAS 1990	N.I.	N.I.
NWAS 1990	N.I.	normal lab., no photoperiod required

7 Hydrogen-ion Concentration at Start of Test

Document	pH, for Test Water Unless Otherwise Specified, and Adjustments
Beak 1988	N.I.
EVS 1989	adjust sample to pH 7.5 if necessary; pH of test water N.I.
B.C. MOE 1990	N.I.
IGATG 1991	N.I.
Dinnel <i>et al.</i> 1987	adjusted if required; levels not indicated
U.S. EPA 1988	N.I.
ASTM 1990	7.8 to 8.1 for Pacific purple s.u., "similar" for other species; adjust c/d water
NCASI 1991, 1992	N.I.
U.S. EPA (Pac. 91)	8.1 $\pm$ 0.1 for c/d water
U.S. EPA (Pac. 92)	N.I.
Kobayashi 1971, 1984	N.I.
S. Calif. Project	Not controlled. Some tests averaged 7.8 to 7.9
Nacci <i>et al.</i> 1986	N.I.
Cherr <i>et al.</i> 1987	N.I.
BML 1991	8.0, adjust both sample and c/d water if necessary, ensure pH is stable
ERCEES 1990	N.I.
MECAS 1990	8.0 $\pm$ 0.2, adjust test solutions as necessary
NWAS 1990	8.0

8 Volume of Test Water, Vessels Used, and Number of Replicates

Document	Volume (mL)	Vessel	Replicates
Beak 1988	5	20-mL scintillation vials, disposable	four
EVS 1989	10	test tubes, 16 × 150 mL with caps	three
B.C. MOE 1990	2	borosilicate glass tubes, disposable	three
IGATG 1991	5	20-mL scintillation vials, disposable	three
Dinnel <i>et al.</i> 1987	10	borosilicate glass test tubes, 16 × 100, disposable, unwashed	≥3
U.S. EPA 1988	5	20-mL scintillation vials, disposable	≥3, normally four
ASTM 1990	N.I.	glass vials, 15 to 22 mL, or other	recommend 4, usually ≥3
NCASI 1991, 1992	2	borosilicate glass culture tubes, 13 × 100 disposable	four
U.S. EPA (Pac. 91)	5	borosilicate glass tubes, 16 × 100 mm	three
U.S. EPA (Pac. 92)	5	disposable glass test tubes, 16 × 100/125 mm	≥3
Kobayashi 1971	N.I.	glass finger bowl, 5 cm diam., 3 cm deep	N.I.
Kobayashi 1984	N.I.	finger bowl filled with test medium	N.I.
S. Calif. Project	50 (sperm) 900 (eggs)	polypropylene cup 1-L beaker	N.I. N.I.
Nacci <i>et al.</i> 1986	10	glass vials	N.I.
Cherr <i>et al.</i> 1987	2	borosilicate culture tubes, 13 × 100 mm	N.I.
BML 1991	2	N.I.	three
ERCEES 1990	10	20-mL scintillation vials	four
MECAS 1990	5	25-mL scintillation vials	≥3
NWAS 1990	10	borosilicate culture tubes, 18 × 150 mm	four

9 Exposure Times for Sperm, for Eggs Plus Sperm, and for Experimental Controls

Document	Sperm exposure	Eggs + Sperm	Control Vessels
Beak 1988	60 min	60 min	4 c/d water
EVS 1989	30 min (s.u.) 60 min (s.d.)	20 min 20 min	3 with c/d water; freshwater samples with duplicate salinity controls made with distilled water, concentrations same as for the sample
B.C. MOE 1990	10 min	10 min	3 seawater
IGATG 1991	60 min	20 min	3 c/d water
Dinnel <i>et al.</i> 1987	60 min	20 min	≥3 c/d water
U.S. EPA 1988	60 min	20 min	≥3, normally 4, c/d water
ASTM 1990	60 min	20 min	c/d water; solvent control if used
NCASI 1991, 1992	10 min	10 min	4 c/d water
U.S. EPA (Pac. 91)	20, 60 min	20, 60 min	diverse, to assess alternate methods in this exploratory round-robin
U.S. EPA (Pac. 92)	60 min	20 min	≥3 c/d water; unfertilized eggs in c/d water and high concentration; optional seawater and receiving water controls; salinity controls if samples <30 or >34 g/kg
Kobayashi 1971	none, sperm and eggs together in 3-min fertilization exposure		yes, assumed in c/d water
Kobayashi 1984	N.I., assume sperm and eggs together in 3-min fertilization exposure, or option with "aged" gametes pre-exposes sperm to test water for 5 min, pre-exposes eggs for several hours		N.I.
S.Calif. Project	15 min	eggs pre-exposed 30 min, then with sperm	4 seawater, plus salinity controls to match effluent concentrations
Nacci <i>et al.</i> 1986	60 min	20 min	N.I.
Cherr <i>et al.</i> 1987	10 min	10 min	yes, details unspecified
BML 1991	10 min	10 min	N.I., assumed c/d water
ERCEES 1990	60 min	20 min	N.I., 4 assumed in c/d water
MECAS 1990	N.I.	N.I.	≥3 seawater
NWAS 1990	60 min	20 min	4 c/d water

10 Stimulation of Spawning and Collecting Gametes

Document	Stimulus Used	Collecting
Beak 1988	0.5 mL of 0.5M KCl	5 mm seawater in petri dishes
EVS 1989	0.5 mL of 0.5M KCl (2nd injection if needed)	c/d water in 150-mL beaker
B.C. MOE 1990	s.u., 1.0 mL of 0.5M KCl s.d., 0.5 mL of 0.5M KCl	as above seawater at 10 °C in 250 mL beaker
IGATG 1991	0.5 mL of 0.5M KCl	5 mm seawater in petri dishes
Dinnel <i>et al.</i> 1987	s.u. 1.0 mL of 0.5M KCl s.d. 0.5 mL	seawater in 100-mL beaker
U.S. EPA 1988	12 volts D.C. for 30 sec	bowl, shallow c/d water, use syringe
ASTM 1990	most species, 0.5 to 1.0 mL of 0.5M KCl, 2nd injection if no spawn in 10 min; use 12 volts D.C. for <i>Arbacia</i>	seawater in small beaker
NCASI 1991, 1992	s.u. 1.0 mL s.d. 0.5 mL of 0.5M KCl	collect with pipet to tubes at 12°C c/d water in 50-mL beaker (s.u. 100-mL beaker)
U.S. EPA (Pac. 91)	0.5 to 1.0 mL of 0.5M KCl, 2nd injection if needed	c/d water in 100-mL beaker
U.S. EPA (Pac. 92)	0.5 mL of 0.5M KCl, 2nd injection if needed	eggs in c/d water in 100-mL beaker, semen "dry"
Kobayashi 1971	KCl injection for ♀	testes removed, "dry" sperm to seawater
Kobayashi 1984	"KCl method"	N.I.
S.Calif. Project	0.5 mL of 0.5M KCl	eggs into seawater in 100-mL beaker, semen "dry" with pipet to tubes at <5°C
Nacci <i>et al.</i> 1986	electrical	N.I. moistened (♂), collect with pipet to vials on ice
Cherr <i>et al.</i> 1987	0.5 mL of 0.5M KCl	seawater in 50-mL beaker
BML 1991	0.5 to 1.0 mL of 0.5M KCl	shake and place on fingerbowl with seawater (♀) or
ERCEES 1990	0.5 mL of 0.5M KCl	in small beaker, "dry" for sperm, water for eggs
MECAS 1990	0.5 mL of 0.5M KCl, 2nd inject'n in 5 min if needed	eggs into 100-mL beaker with 20 mL water, sperm "dry" with syringe to vial on ice
NWAS 1990	s.u. 1.0 mL of 0.5M KCl, s.d. 0.5 mL	on empty 100-mL beaker, collect eggs to cold c/d water, semen "dry" with pipet to cooled test tube

11 Holding Gametes

Document	Conditions and Limitations for Holding
Beak 1988	sperm composited from several males
EVS 1989	s.u. sperm on ice, wash eggs 3 times, pool gametes from ♂, ♀
B.C. MOE 1990	sperm composited from ≥ 2 males, used ≤ 4 h, eggs stored ≤ 24 h
IGATG 1991	composite sperm, hold on ice, use ≤ 20 min, eggs from 4 animals
Dinnel <i>et al.</i> 1987	sperm activation for ≤ 1.5 h did not affect test, wash eggs 3 times, compositing optional
U.S. EPA 1988	sperm used in < 1 h, kept on ice, eggs keep several hours at lab. temperature
ASTM 1990	sperm in cool seawater keep several hours, keep "dry" and refrigerated for many hours, rinse eggs 2 or 3 times, keep sperm separate and use block design for test or composite
NCASI 1991, 1992	sperm usually ≤ 1 h, eggs normally ≤ 2 h, hold at 12°C
U.S. EPA (Pac. 91)	collect for ≤ 30 min, wash eggs twice, composite sperm
U.S. EPA (Pac. 92)	collect for ≤ 30 min, wash eggs twice, store in water at standard strength, sperm in separate vials on ice and use in ≤ 4 h
Kobayashi 1971	use ≤ 1 h
Kobayashi 1984	use gametes ≤ 1 h, wash eggs several times
S. Calif. Project	"dry" semen stored at $< 5^\circ\text{C}$, pool eggs from 6 ♀, wash twice
Nacci <i>et al.</i> 1986	N.I.
Cherr <i>et al.</i> 1987	gametes on ice for ≤ 2 h
BML 1991	eggs and "dry" sperm to vials on ice, wash eggs twice
ERCEES 1990	pool sperm, eggs
MECAS 1990	"dry" sperm to vial on ice, wash eggs twice, hold in dark at 12°C
NWAS 1990	"dry" semen to refrigerated tube, wash eggs twice and use fresh

12 Numbers of Gametes Used Per Test Vessel and Sperm-to-egg Ratios

Document	Sperm/Vessel	Eggs/Vessel	Sperm:Egg Ratio
Beak 1988	7 or 5 million?	2000	2500 or 3500:1 ?
EVS 1989	s.u. 4 million	2000	2000:1
	s.d. 2.4 million	2000	1200:1
B.C. MOE 1990	N.I.	500	N.I.
IGATG 1991	~ 5 million	2000	~2500:1
Dinnel <i>et al.</i> 1987	various	2000	determine appropriate ratio, commonly purple s.u. 200:1, red s.u. 1000:1, green s.u. 2000. s.d. (<i>D. excentricus</i>) 1200:1
U.S. EPA 1988	5 million	2000	2500:1
ASTM 1990	empirical to give 70% to 90% fertilization	200/mL of test solution	commonly 200:1 for purple s.u., s.d. 1200:1, others 2000 to 2500:1
NCASI 1991, 1992	s.d. 20 000 to 60 000	500	40:1 to 120:1
	s.u. empirical	500	determine appropriate ratio
U.S. EPA (Pac. 91)	various	1120?	various, to assess methods
U.S. EPA (Pac. 92)	560 000	1120	500:1 (fixed ratio)
Kobayashi 1971	N.I.	N.I.	N.I.
Kobayashi 1984	N.I.	N.I.	N.I.
S.Calif. Project	N.I. (1.2 mL of standard preparation)	31 500	N.I.
Nacci <i>et al.</i> 1986	0.1 million	1000	100:1 (authors say 1000:1)
Cherr <i>et al.</i> 1987	0.5 million	500	1000:1
BML 1991	N.I. (0.1 mL "dry")	N.I. (0.1 mL)	1000:1
ERCEES 1990	empirical	2000	determine appropriate ratio
MECAS 1990	1 million?	empirical	determine ratio needed for 70% to 90% fertilization
NWAS 1990	empirical	2000	determine ratio needed for 70% to 90% fertilization, commonly ratios from 200:1 to 2000:1

13 Adjustment of Results for Degree of Fertilization in Controls *

Document	Method of Adjustment
Beak 1988	Abbott's formula
EVS 1989	Abbott's formula
B.C. MOE 1990	Abbott's formula
IGATG 1991	Abbott's formula:** $A = (O - C) \cdot (100) / (100 - C)$
Dinnel <i>et al.</i> 1987	Abbott's formula
U.S. EPA 1988	Abbott's formula
ASTM 1990	"Adjusted percent Fertilization" = $AF = 100 \cdot OF/CF$ ** [symbols changed, this gives same result as Abbott's formula, but is calculated for fertilization]
NCASI 1991, 1992	N.I.
U.S. EPA (Pac. 91)	N.I.
U.S. EPA (Pac. 92)	as in U.S. EPA (1988)
Kobayashi 1971	N.I.
Kobayashi 1984	N.I.
S.Calif. Project	IC50 not mentioned as a statistic to be estimated
Nacci <i>et al.</i> 1986	N.I.
Cherr <i>et al.</i> 1987	"normalized" for control fertilizations, method not stated
BML 1991	N.I.
ERCEES 1990	N.I.
MECAS 1990	N.I.
NWAS 1990	Abbott's formula

* The kinds of adjustment shown here are not used to estimate NOEC/LOEC; the unmodified (i.e., raw) values for percent fertilization are used in those calculations. The adjustments shown above produce the numerical equivalents of the Percent Reduction in Fertilization calculated in the present document (Subsection 4.5.3) as a preliminary to estimating an ICp.

** A = Adjusted percentage of unfertilized eggs for the exposure in a given test vessel
 O = Observed percentage of unfertilized eggs for the test exposure in a given test vessel
 C = Control percentage of unfertilized eggs in dilution/control water
 AF = Adjusted percentage of **fertilized** eggs in a given test vessel
 OF = Observed percentage of **fertilized** eggs in a given test vessel
 CF = Control percentage of **fertilized** eggs in dilution/control water

14 Requirements for Valid Test

Document	Percent Fertilization in Control	Other Requirements
Beak 1988	N.I.	
EVS 1989	N.I.	
B.C. MOE 1990	N.I.	
IGATG 1991	N.I.	
Dinnel <i>et al.</i> 1987	≥50	
U.S. EPA 1988	≥70 (>90 might mask toxicity)	
ASTM 1990	≥50, desirable 70 to 90, best 80 to 95	
NCASI 1991, 1992	50 to 100 acceptable, 50 to 90 preferred	
U.S. EPA (Pac. 91)	desirable to attain 80 to 95	
U.S. EPA (Pac. 92)	fertilization ≥50 in control; sperm concentration within a factor of two of desired; essentially zero fertilization in egg controls in c/d water and effluent	
Kobayashi 1971	N.I.	
Kobayashi 1984	pre-test check ≥85 ("aged gametes" ≥91)	membrane to elevate within 3 min of fertilization
S. Calif. Project	N.I.	
Nacci <i>et al.</i> 1986	≥60, ≤90	
Cherr <i>et al.</i> 1987	N.I.	
BML 1991	N.I.	
ERCEES 1990	≥70, ≤90	positive and logical dose-effect curve; physical and chemical requirements met
MECAS 1990	N.I.	
NWAS 1990	≥70, ≤90	

15 Reference Toxicant

Document	Chemical	Required?	Test Type or Endpoint*
Beak 1988	N.I.		
EVS 1989	sodium dodecyl sulphate	yes	in duplicate, 5 concentrations 1.0 to 10 mg/L
B.C. MOE 1990	N.I.		
IGATG 1991	cadmium chloride	no	
Dinnel <i>et al.</i> 1987	silver	no	
U.S. EPA 1988	copper sulphate	yes	with each batch of gametes
ASTM 1990	N.I.	no	"might assess sensitivity of a spawning"
NCASI 1991, 1992	N.I.		
U.S. EPA (Pac. 91)	copper	no?	
U.S. EPA (Pac. 92)	copper, sodium dodecyl sulphate, or other	yes	with each set of tests
Kobayashi 1971	N.I.		
Kobayashi 1984	N.I.		
S. Calif. Project	N.I.		
Nacci <i>et al.</i> 1986	N.I.		
Cherr <i>et al.</i> 1987	sodium azide	no	
BML 1991	N.I.		
ERCEES 1990	N.I.		
MECAS 1990	N.I.		
NWAS 1990	sodium azide	yes	concurrent with main test

* Unless otherwise indicated, the test is the standard type with the endpoints used in the main test, and estimation of IC_p and NOEC/LOEC.

Appendix D

Bibliography. Additional Papers Directly Relevant to Canadian Echinoid Fertilization Assay

This list could assist laboratories wishing to enter the wider literature on echinoid testing. Many of these publications contain data on toxic concentrations of various pollutants to echinoid gametes, or compare findings for other stages of development or other organisms. Some annotations have been added in square brackets.

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Appendix E

Logarithmic Series of Concentrations Suitable for Toxicity Tests*

Column (Number of concentrations between 100 and 10, or between 10 and 1) **

1	2	3	4	5	6	7
100	100	100	100	100	100	100
32	46	56	63	68	72	75
10	22	32	40	46	52	56
3.2	10	18	25	32	37	42
1.0	4.6	10	16	22	27	32
	2.2	5.6	10	15	19	24
	1.0	3.2	6.3	10	14	18
		1.8	4.0	6.8	10	13
		1.0	2.5	4.6	7.2	10
			1.6	3.2	5.2	7.5
			1.0	2.2	3.7	5.6
				1.5	2.7	4.2
				1.0	1.9	3.2
					1.4	2.4
					1.0	1.8
						1.3
						1.0

* Modified from Rochinni *et al.* (1982).

** A series of five or more successive concentrations should be chosen from a column. Mid-points between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed as percentage by volume or weight, mg/L, or µg/L. As necessary, values could be multiplied or divided by any power of 10. Column 1 might be used if there was considerable uncertainty about the degree of toxicity. More widely spaced concentrations (differing by a factor <0.3) should not be used. For effluent testing, there is seldom much gain in precision by selecting concentrations from a column to the right of column 3; the finer gradations of columns 4 to 7 might occasionally be useful for testing chemicals that have an abrupt threshold of effect.